CONTROL OF THE EBV GROWTH TRANSFORMATION PROGRAMME: THE IMPORTANCE OF THE BAMHI W REPEATS

by

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Abstract

Epstein-Barr virus (EBV), a human gammaherpesvirus, possesses a unique set of latent genes whose constitutive expression in B cells leads to cell growth transformation. The initiation of this B-cell growth transformation programme depends on the activation of a viral promoter, Wp, present in each tandemly arrayed BamHI W repeat of the EBV genome. In order to examine the role of the BamHI W region in B cell infection and growth transformation, we constructed a series of recombinant EBVs carrying different numbers of BamHI W repeats and carried out B cell infection experiments. We concluded that EBV requires at least 2 copies of BamHI W repeats to be able to activate transcription and transformation in resting B cells *in vitro*. At least 5 copies of BamHI W were required for optimal transcription and transformation; while increasing the number beyond 5 copies had no further effect. Experiments to try to rescue the impaired virus indicated that the expression of sufficient levels of EBNA-LP and EBNA2 from Wp are the key determinants of virus-driven B cell transformation. We believe that EBV has evolved to contain multiple copies of BamHI W repeats to ensure high levels of Wp-initiated transcripts immediately post infection.

For Mum, Dad, Chris and Tina

獻給我敬愛的爸爸 媽媽 及仁 和 協正

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ABBREVIATIONS

A	adenine
ACV	acycloguanosine
AIDS	acquired immunodeficiency syndrome
AMV	avian myeloblastosis virus
AP1	activating protein 1
APS	ammonium persulphate
ATF	activating transcription factor
ATP	adenosine triphosphate
AUF1	A+U-rich-element RNA-binding factor
β2m	beta-2-microglobulin
BAC	bacterial artificial chromosome
BARF0	BamHI A rightward frame 0
BART	BamHI A rightward transcript
BCR	B cell recepotor
BD	bound
BL	Burkitt's lymphoma
bp	base pair
BSAP	B cell-specific activator protein
C	carboxy
C	cytosine
CAP	calf alkaline phosphatase
CBF	Cp binding factor
CBP	CREB-binding protein
CD	complementarity-determining
cDNA	complementary deoxyribonucleic acid
C/EBP	CCAAT/enhancer binding protein
ChIP	chromatin immunoprecipitation
CMV	cytomegalovirus
Cp	*Bam*HI C promoter
CRE	cAMP response element
CREB	CRE-binding protein
CST	complementary strand transcript
Ct	threshold cycle
CTAR	carboxy-terminal activating region
CTL	cytotoxic T lymphocyte
D	diversity segment
DAPI	4',6'-diamidino-2-phenylindole
DME	Dulbecco's Modified Eagle's
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DNMT	DNA methyltransferase
dNTP	deoxyribonucleoside triphosphate

ds	double-stranded
DS	dyad symmetry
DTT	dithiothreitol
EBER	EBV encoded RNA
eBL	endemic Burkitt's lymphoma
EBNA	EBV nuclear antigen
EBNA-LP	EBNA-leader protein
EBV	Epstein-Barr virus
ECL	enhanced chemiluminescence
EDTA	diaminoethanetetraacetic acid
EGFR	epidermal growth factor receptor
EHV	equine herpes virus
EIHV	elephantid herpesvirus
EMSA	electrophoretic mobility shift assay
ERK	extracellular signal-related kinase
FACS	fluorescence activated cell sorter
FAM	6-carboxy-fluorescein
FCS	foetal calf serum
FD	frequency domain
Fp	*Bam*HI F promoter
FR	family of repeats
G	guanine
GaHV	gallid herpesvirus
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GFP	green fluorescent protein
gp	glycoprotein
GRE	glucocorticoid response element
HAT	histone acetyl transferase
HCl	hydrochloric acid
HCMV	human cytomegalovirus
HD	Hodgkin's disease
HDAC	histone deacetylase
HEPES	N-[2-Hydroxyethyl] piperazine-N'-[2-ethanesulphonic acid]
HHV	human herpes virus
HIV	human immunodeficiency virus
HMT	histone methyltransferase
hnRNP	heterogeneous nuclear ribonucleoprotein
HP1	heterochromatin protein 1
HRS	Hodgkin and Reed-Sternberg
hSNF	human sucrose non-fermenting
HSP	heat shock protein
HSV-1	herpes simplex virus type 1
ICF	immunodeficiency, centromere instability, facial anomalies
IFN	interferon
Ig	immunoglobulin

IL	interleukin
IM	infectious mononucleosis
IP	immunoprecipitation
IPTG	isopropyl-β-D-thiogalactoside
IR	internal repeat
IRF	interferon regulatory factor
ISRE	interferon-stimulated response element
ITAM	immunoreceptor tyrosine-based activation motif
J	joining segment
JAK	janus kinase
JNK	c-Jun N-terminal kinase
K	lysine
kbp	kilobase pair
KCl	potassium chloride
kDa	kilodalton
KSHV	Kaposi's sarcoma associated herpesvirus
LAT	latency associated transcript
L-Broth	Lennox Broth
LCL	lymphoblastoid cell line
LCV	Lymphocryptovirus
LiCl	lithium chloride
LMP	latent membrane protein
LMP1p	LMP1 promoter
LRS	LMP1 transcription regulatory sequence
LSD	lysine-specific demethylase
M	methylated
mAb	monoclonal antibody
MAPK	mitogen-activated protein kinase
MBD	methyl-CpG binding domain
MCMV	mouse cytomegalovirus
MeCP	methyl-CpG binding protein
$MgCl_2$	magnesium chloride
$MgSO_4$	magnesium sulphate
MHC	major histocompatibility complex
MIEP	major immediate early gene promoter
miRNA	micro RNA
m.o.i.	multiplicity of infection
mRNA	messenger ribonucleic acid
MSP	methylation-specific PCR
N	amino
NaCl	sodium chloride
N-ChIP	native chromatin immunoprecipitation
NCoR	nuclear receptor corepressor
n.d.	not determined
NF-κB	nuclear factor kappa B

$Notch^{IC}$	intracellular Notch
NP-40	nonidet P-40
NPC	nasopharyngeal carcinoma
NuRD	nucleosome remodelling and deacetylase
ORF	open reading frame
OriP	origin of plasmid replication
OriLyt	origin of lytic replication
PBMC	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PCAF	p300/CBP-associated factor
PCR	polymerase chain reaction
Peg3	paternally expressed gene 3
PIPES	piperazine-1,4-bis[2-ethanesulphonic acid]
PKR	RNA-activated protein kinase
PMSF	phenylmethyl sulphonyl fluoride
PNK	polynucleotide kinase
Pol	EBV DNA polymerase
POU	Pit, Oct, Unc
PRMT	protein arginine methyltransferase
PTLD	post-transplant lymphoproliferative disease
Qp	*Bam*HI Q promoter
Q-PCR	quantitative PCR
RNA	ribonucleic acid
Rb	retinoblastoma protein
RBP-Jκ	recombination binding protein J kappa
RFX	regulatory factor X
RNP	ribonucleoprotein
Rp	*Bam*HI R promoter (BRLF1 promoter)
RT	reverse transcription
RT	room temperature
RT-PCR	reverse transcriptase PCR
RV	rhadinovirus
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SIN	SWI/SNF-independent
siRNA	small inhibitory RNA
SKIP	ski interacting protein
SNF	sucrose non-fermenting
Sp1	promoter-specific transcription factor
ssDNA	single-stranded DNA
STAT	signalling transducer and activator of transcription
SUMO	small ubiquitin-like modifier
Suv	suppression of variegation
SWI	switching (yeast mating type locus)
Syk	spleen tyrosine kinase

T	thymine
TAF	TATA-binding protein associated factor
TAMRA	6-carboxy-tetramethylrhodamine
TCA	trichloroacetic acid
TEMED	N,N,N',N'-tetramethylethylenediamine
TFIIB	transcription factor II B
TNF	tumour necrosis factor
TR	terminal repeat
TRADD	TNF receptor-associated death domain
TRAF	TNF receptor-associated factor
Tween	polyoxyethylene sorbitan monolaurate
U	unmethylated
U	uracil
UAS	upstream activating sequence
UM	unfractionated mononuclear
V	variable segment
VAHS	virus-associated haemophagocytic syndrome
VCA	viral capsid antigen
VDJ	variable-diversity-joining
VZV	varicella zoster virus
WB	Western blotting
WCL	whole cell lysate
Wp	*Bam*HI W promoter
X-ChIP	cross-linked chromatin immunoprecipitation
X-gal	5-bromo-4-chloro-3-indoyl-β-D-galactoside
YY1	Yin yang 1
Zp	*Bam*HI Z promoter (BZLF1 promoter)

Chapter 1. Introduction

1.1 Epstein-Barr virus (EBV) history

EBV was discovered as a result of the pioneering work in the 1950s, by Denis Burkitt, a surgeon working in East Africa (Burkitt, 1958). Burkitt spent many years mapping the distribution of a fast-growing tumour which affected the jaws of young African children. This tumour was previously undescribed, but is now classified as a lymphoma. Burkitt noted that this tumour was most common in children living in areas where malaria was holoendemic and speculated that an infectious arthropod-borne agent may be involved in its etiology (Burkitt, 1962b, Burkitt, 1962a). A collaboration was set up between Burkitt and pathologist Tony Epstein, where BL biopsy samples were transported from Uganda to England. Epstein and his PhD student, Yvonne Barr, established cell lines from the biopsy samples and, by electron microscopy, identified the presence of herpesvirus-like particles in some of the cells (Epstein et al., 1964, Epstein and Barr, 1964). The absence of biological activities associated with the then known human herpesviruses identified EBV as a new member of the herpesvirus family (Epstein et al., 1965, Henle and Henle, 1966b, Henle and Henle, 1966a).

More than 95% of adults worldwide are infected with EBV (Henle et al., 1969, Henie et al., 1974). In the majority of cases, a harmless life-long infection is established in the B cells, with periodic shedding of the virus into the saliva and then spread through saliva. Importantly EBV from BL cells was shown to transform resting B lymphocytes *in vitro*

leading to the outgrowth of B lymphoblastoid cell lines (LCLs), thus establishing EBV as a B lymphotropic transforming virus (Henle et al., 1967).

1.2 Classification of Herpesviruses

Like other herperviruses, EBV has a toroid-shaped protein core wrapped with a 184 kilobase pair linear, double-stranded DNA, encased by a nucleocapsid with 162 capsomers, a protein tegument between the nucleocapsid and the envelope, and an outer envelope with external glycoprotein spikes (Epstein et al., 1965, Pope et al., 1968). EBV (also named as HHV4) is a member of the *Herpesviridae*. There are three sub-families of the *Herpesviridae*, -alpha, beta and gamma herpesviruses, which were originally classified by their biologic properties, such as host range, duration of reproduction cycle, cytopathology and characteristics of latent infection. This classification has now been amended on the basis of genome organisation and homology.

Alphaherpesvirinae

The members of this subfamily were classified on the basis of a variable host range, relatively short reproductive cycle, rapid spread in culture, efficient destruction of infected cells, and the capacity to establish primarily latent infections. The subfamily contains the genera *Simplexvirus* (prototype: HSV-1), *Varicellovirus* (prototype: VZV), *Mardivirus* (prototype: GaHV-2), and *Iltovirus* (prototype: GaHV-1). *Simplexviruses* and *Varicelloviruses* have mammalian hosts, and GaHV-1 and GaHV-2 have avian hosts.

Betaherpesvirinae

Unlike Alphaherpesvirinae, the characteristics of the members of this family are a restricted host range; all the members of this family are restricted to mammalian hosts. The

reproductive cycle is long and the infection progresses slowly in culture. The virus can be maintained in latent form in secretory glands, lymphoreticular cells, kidneys and other tissues. This subfamily contains the genera *Cytomegalovirus* (prototype: HCMV), *Muromegalovirus* (prototype: MCMV), *Roseolovirus* (prototype: HHV-6), and the proposed *Proboscivirus* (prototype: EIHV-1).

Gammaherpesvirinae

The experimental host range of the members of the subfamily *Gammaherpesvirinae* is limited to the family or order to which the natural host belongs. Most of these viruses are characterised by the establishment of lifelong infection in their host, association with cellular transformation and the development of malignancies (Simas and Efstathiou, 1998). *In vitro*, all members replicate in lymphocytes, and some also cause lytic infections in some types of epitheloid and fibroblastic cells. Viruses in this group are usually specific for either T or B lymphocytes. Members of the *Gammaherpesvirinae* subfamily are divided into two genera: gamma-1 herpesviruses, or *Lymphocryptovirus* (LCV) which are B-lymphotropic viruses, e.g., Epstein-Barr virus and genetically related viruses of old world primates, and the gamma-2 herpes viruses, or *rhadinoviruses* (RVs), e.g., herpes virus saimiri (HVS) of new world monkeys and HHV-8 of humans (Simas and Efstathiou, 1998). Many old world and some new world primate species have endemic LCV, in contrast, RVs are endemic not only in most primate species but also in some subprimate mammalian species, for example, MHV68 and related viruses of wild mice (Kieff and Rickinson, 2007). EBV is the only human LCV, and Kaposi's sarcoma associated herpesvirus (KSHV/HHV8) is the only human RV.

1.3 Herpesviruses genomes

Herpesviruses genomes can be divided into six groups, A-F (Figure 1.1.) The class A genome consists of a unique sequence flanked by terminal repeats (TR). It is represented among *Betaherpesvirinae* (HHV6 and HHV7) and one member of the *Gammaherpesvirinae* (EHV2). Class B genomes have directly repeated (DR) sequences at the termini, but these consist of variable copy numbers of a tandemly repeated sequence of 0.8-2.3 kbp. This arrangement characterises most Gammaherpesvirinae in the Rhadinovirus genus, such as HVS and HHV8. Group C genomes, exemplified by EBV represent another derivative of class B, in which an internal set of direct repeat is present but is unrelated to the terminal set. Class D genomes, exemplified by VZV contain two unique regions (U_L and U_S), one internal repeat region (IR) and one terminal repeat (TR). In Class E, exemplified by HSV1, sequences from both termini are repeated in an inverted orientation and juxtaposed internally dividing the genomes into two components, each of which consists of unique sequences flanked by inverted repeats. In Class F exemplified by tupaia herpesvirus, the terminal sequences are not identical and are not repeated either directly or in an inverted orientation.

1.4 The EBV genome

EBV was the first herpesvirus to have its complete genome cloned and sequenced (Dambaugh et al., 1980, Arrand et al., 1981, Baer et al., 1984). This was done using *Bam*HI restriction fragments from the B95.8 EBV strain, derived from an infectious mononucleosis (IM) patient isolate and passaged in marmoset B cells *in vitro*. Figure 1.2 shows a schematic of the circular EBV genome. Open reading frames (ORFs) are named relative to

the *Bam*HI fragment, direction of transcription and position relative to other ORFs. Promoters are also named according to their location in a particular *Bam*HI fragment. For example, BHRF1 is the first rightward ORF located on the *Bam*HI H fragment and Wp is the promoter in the *Bam*HI W fragment.

EBV contains several repeat regions within its genome. The terminal repeats (TRs) are tandem, 500kb direct repeats of the same sequence at both termini of the linear genome, which mediate circularisation of the viral DNA into episomes (Kintner and Sugden, 1979). The precise number of TRs is determined during virus replication and this provides a useful indicator of cell clonality, as latently infected progeny cells will retain the same number of TRs as the parental cells (Raab-Traub and Flynn, 1986). The large internal repeat 1 (IR1) within the EBV genome comprises 3 to 12 tandem reiterations of a 3kb, direct repeat, which comprises numerous repeats of a 3072bp BamHI W region containing one of the EBNA promoters, Wp. The number of BamHI W repeats, and therefore the number of Wp copies, also varies between EBV strains.

All EBV strains have essentially the same genome organisation and most parts of the genomes are highly conserved. However, there are two EBV types circulating in most populations, type 1 and type 2, reflecting linked polymorphisms in the latent genes encoding EBV nuclear antigens (EBNAs)-LP, 2, 3A, 3B and 3C (Sixbey et al., 1989, Sample et al., 1990). Epidemiologically, type 1 strains are far more common in most populations but in equatorial Africa and New Guinea, type 2 appears to be more prevalent although type 1 strains are still in the majority.

1.5 *In vivo* EBV infection

1.5.1 Primary infection

EBV is naturally transmitted via the salivary route and infection results in life-long EBV persistence. Primary EBV infection usually occurs asymptomatically during early childhood (Henle and Henle, 1970). However, if infection is delayed until adolescence, as is often the case in the Western world, a self-limiting lymphoproliferative disease termed infectious mononucleosis (IM) can ensue (Henle et al., 1968). Symptoms can range from mild transient fever to several weeks of pharyngitis, lymphodenopathy and general malaise, which are mainly caused by the activation and proliferation of the host CD8+ T cells in response to viral infection.

1.5.2 Lytic infection at Oropharyngeal sites

Figure 1.3 outlines the sites of EBV infection and the type of infection (latent or lytic) established within each cell population. Primary infection normally takes place in the muscosal tissues of the oropharynx, but the initial target cell for infection, either an epithelial cell or a B cell, is still not clear. One theory suggests that EBV first infects oropharyngeal epithelial cells, which then undergo lytic cycle to generate virions which can infect B cells. Certainly high levels of infectious virus have been detected in throat washing of IM patients (Gerber et al., 1972, Yao et al., 1985) and the presence of the virus has been reported in desquamated oropharyngeal epithelial cells of such patients (Sixbey et al., 1984). In addition, a recent report described the presence of EBV DNA, mRNA and protein in a small subset of tonsillar epithelial cells from healthy, EBV-positive donors (Pegtel et al., 2004). Oral hairy leukoplakia in immunocompromised patients is characterised by

productive EBV replication within the upper-layer epithelial cells undergoing terminal differentiation, apoptosis and desquamation, strongly suggesting that EBV replication *in vivo* is dependent upon the differentiation state of the epithelial cells (Becker et al., 1991, Walling et al., 2004). In addition, EBV replication has been reported in normal tongue epithelium, suggesting the tongue may be a source of EBV secreted into saliva (Walling et al., 2001). Alternatively, it is suggested that B cells may be directly infected, and crypt structures in the tonsil formed by invaginations of the tonsillar lymphoepithelium might allow direct access of EBV-containing saliva to B cells in lymphoid tissue below (Perry et al., 1988). Thus patients with B-cell-deficient, X-linked agammaglobulinemia show no evidence of even transient EBV infection in the throat implying that acquisition of EBV by the naïve host depends on initial B-cell infection (Faulkner et al., 1999). A recent finding that epithelial cell infection is rendered more than 1000 times more efficient if the virus is first bound to the surface of a resting B cell (Shannon-Lowe et al., 2006), suggests EBV might access epithelium not through an intermediate round of replication in B cells but through binding to the resting B cell surface and using this surface as a transfer vehicle. Therefore binding of orally transmitted EBV to B cells within the lymphoepithelium of the oropharynx may be important for EBV entry into permissive epithelium.

1.5.3 Latent infection: Colonization of the B cell system

Orally transmitted virus establishes replicative foci within the oropharynx, and at the same time, the virus begins to colonise the B cell system. However, the events involved in virus colonisation of the B cell system during primary infection are still poorly understood. Viral transcripts associated with growth transformation are detectable in peripheral blood

mononuclear cells (PBMCs) and tonsils of infectious mononucleosis (IM) patients, indicating that primary infection is associated with a transient virus-driven expansion of the infected B-cell pool through a growth transforming programme (Tierney et al., 1994, Niedobitek et al., 1997a).

The human circulating B-cell pool contains 3 main populations: (1) antigen-inexperienced naïve cells (IgD+CD27-), (2) isotype-switched memory cells (IgD-CD27+), and (3) nonswitched memory cells (IgD+CD27+). In the blood of healthy virus carriers and IM patients, EBV-infected cells were found to be sequestered in the IgD- and in the CD27+fractions, consistent with selective colonization of the IgD-CD27+ memory cells (Hochberg et al., 2004b, Babcock et al., 1998, Joseph et al., 2000).

This has given rise to a model for EBV colonization of the B-cell pool in which virus first targets naïve B cells as a growth-transforming infection (Latency III, see later) that, mimicking antigen stimulation, drives these cells down the physiologic, GC-dependent route into memory. In GC B cells, a more restricted EBV gene expression pattern is detected (Latency II, see later), in which only EBNA1, LMP1 and LMP2A proteins are expressed. The clonal expansion in the germinal centre increases the size of the pool of EBV-infected B cells. These GC B cells will then differentiate into resting memory B cells, which are the long-term reservoir of EBV. In this process, antigen activates B cells to become blasts that enter primary follicles, proliferate, and alter their Ig genes through isotype switching and hypermutation (Thorley-Lawson, 2001). As memory B cells are resting and long-lived, EBV can persist in these cells for a long time without needing to

express any viral genes (Latency 0). This allows virus to persist in memory B cells as an immunologically silent infection in the face of the host T cell response. Limiting dilution RT-PCR analysis for study at the single-cell level has shown that more than 90% of EBV-infected resting memory cells express no protein-coding latent gene transcripts whatsoever, and that the genome maintenance protein EBNA1 is occasionally detected, presumably to preserve the EBV episome when the cells divide as part of the normal physiologic turnover of memory B cells (Hochberg et al., 2004a).

Although the above model is supported by numerous studies, there are some arguments. *In vitro*, naïve and memory B cells are equally susceptible to EBV infection (Ehlin-Henriksson et al., 2003) and also in acute IM patients, EBV-infected B cells in tonsillar tissue are almost all in exrafollicular areas and are rarely detected in GCs. A different model of B-cell colonization, envisaging a GC-independent route into memory, is based largely on evidence from IM tonsil sections (Kuppers, 2003). There the EBV-infected cells population in extrafollicular areas is dominated by clones with stable memory Ig genotypes without ongoing hypermutation (Anagnostopoulos et al., 1995, Niedobitek et al., 1997a, Kurth et al., 2000). This model proposes that, instead of infecting naïve B cells, EBV can directly infect memory B cells during infectious mononucleosis in the oropharynx. Recently, Chaganti and colleagues have showed that EBV was detectable in the nonswitched memory pool both in IM patients undergoing primary infection and in most long-term virus carriers by qPCR. In IM tonsillar cells, viral loads were concentrated in B cells with the CD38 marker of GC origin but lacking the other GC markers, CD10 and CD77 (Chaganti et al., 2008). These findings suggest that EBV infection in IM tonsils

involves extrafollicular B cells expressing CD38 as an activation antigen and not as a marker of ectopic GC activity.

Virus replication in the oropharynx and virus–driven proliferation and expansion of the B cell pool initiate a T cell response and EBV-specific $CD8^+$ CTLs are induced into rapid proliferation. Despite the strong CTL response, the virus establishes a lifelong persistence and remains detectable at very low levels both as infectious virus in throat washings and as a latent infection (largely latency 0, see later) in the B cell pool (Yao et al., 1985, Miller et al., 1973, Nilsson et al., 1971).

1.5.4 T cell responses to EBV

The immune response to EBV in acute IM patients represents one of the extreme examples of a highly activated response to a virus. The ability to follow acute EBV disease in IM patients alongside chronic infection in healthy carriers has allowed in-depth study of the T cell response to EBV infection (Hislop and Sabbah, 2008). The most important feature of the immune response in IM patients is the dramatic expansion of CD8+ T cells seen in the peripheral circulation, where up to 80% of the PBMC are CD8+ T cells. These expanded CD8+ populations are specific for both latent and lytic cycle antigens. Although lytic-specific CD8+ T cells have been shown to dominate the population, expanded CD8+ populations with latent specificities typically to EBNA3-derived epitope are also detectable (Callan et al., 1998, Catalina et al., 2001, Hislop et al., 2002, Hislop et al., 2005, Steven et al., 1996). Following the resolution of acute IM disease, there is a rapid contraction of the majority of the EBV-specific CD8+ T cell population (Catalina et al., 2001, Hislop et al.,

2002, Hislop and Sabbah, 2008). A similar picture is seen when analyzing the specificity of EBV-specific CD8+ T cell memory responses in healthy donors, although the relative frequency of these T cells is generally much lower than seen in IM patients (Bihl et al., 2006, Benninger-Doring et al., 1999, Saulquin et al., 2000).

No CD4+ T cell expansion has been detected even during the early stages of EBV acute infection (Maini et al., 2000). However, EBV specific CD4+ T cell memory response are detectable in virus carriers. Responses to a wide range of latent and lytic proteins exist, but the frequencies are lower than CD8+ T cell's.

1.6 EBV associated malignancies

1.6.1 Post-transplant lymphoproliferative disease (PTLD) (Latency III)

Patients receiving organ or bone marrow transplantation are usually treated with immunosuppressive drugs to avoid graft rejection. The link between immunosuppressive therapy and lymphoma risk is now well documented from epidemiologic studies on large cohorts of transplant recipients (Craig et al., 1993) and immunosuppressed transplant patients have been shown to be at increased risk of developing a particular spectrum of EBV-associated B lymphoproliferative lesions/lymphomas termed PTLD (Penn, 1979). The association of EBV with PTLD is well recognised (Thomas et al., 1990). Patients developing PTLD tend to present with fever, lymphadenopathy or nonspecific symptoms and only occasionally, with acute systemic disease. Usually this disease arises within the first year after transplantation and presents as multifocal lesions within either lymphoid tissue, the central nervous system (CNS), the liver or the transplanted organ (Nalesnik,

1998). PTLD often derives from EBV-infected donor B cells present with the graft itself. Therefore, PTLD risk can be markedly reduced by protocols that also deplete the graft of B cells (Hale and Waldmann, 1998). In addition, measuring and monitoring increases in EBV loads in the PBMCs of transplant patients may serve as a useful diagnostic tool to predict the likelihood of PTLD onset (Savoie et al., 1994, Rooney et al., 1995, Bai et al., 1997, Rowe et al., 1997, Lucas et al., 1998, Kimura et al., 1999, Baldanti et al., 2000). Most early onset PTLD tumours display a broad pattern of EBV latent gene expression, with expression of EBNA1, EBNA2 and LMP1, consistent with *in vivo* outgrowth of EBV infected cells in the absence of CTL responses (Young et al., 1989, Thomas et al., 1990, Rea et al., 1994, Delecluse et al., 1995, Oudejans et al., 1995).

There is therefore a strong parallel between viral gene expression in PTLD (Young et al., 1989, Gratama et al., 1991) and that seen in *in vitro* infected LCLs. Indeed studies on LCLs have been the key to defining the growth transformation Latency III pattern of infection. As shown in Figure 1.4, latency III, infected cells express all six nuclear antigens, EBNAs 1, 2, -LP, 3A, 3B and 3C from Wp- or Cp-initiated transcripts and the membrane proteins LMPs 1, 2A and 2B transcripts initiating from their own promoters. In addition, the most abundant EBV-encoded RNAs, the EBERs and the highly spliced Bam A rightward transcripts (Brooks et al., 1993) are also transcribed.

These viral gene products cause the resting B lymphocytes to enter cycle and continuously proliferate. Most infected B cells enter the first round of cell DNA synthesis 48-72 hours post-infection (Alfieri et al., 1991, Moss et al., 1981, Moss et al., 1986). Such EBV infected

lymphoblasts in many ways are similar to lymphocytes proliferating in response to antigen, mitogen or IL4 *in vitro*; both show high level expression of activation and adhesion markers, secretion of immunoglobulin (Ig) and the acquisition of efficient antigen-processing function (Wang et al., 1988, Wang et al., 1990a, Peng and Lundgren, 1992, Peng and Lundgren, 1993, Rowe et al., 1995).

Other PTLDs clearly show a more restricted pattern of gene expression, with only EBNA1 protein detectable (Delecluse et al., 1995, Oudejans et al., 1995). It is possible that this latter group of tumours, which tend on average to be later onset post-transplant, have acquired genetic abnormalities over time and are less reliant on EBV for growth transformation.

1.6.2 Burkitt lymphoma (BL) (Latency I)

Burkitt lymphoma (BL) occurs in three epidemiologically distinct forms, endemic, sporadic and AIDS-associated forms. Endemic BL was first described by Denis Burkitt as a sarcoma of the jaw (Burkitt, 1958). BL is the most common cancer of childhood in equatorial Africa and Papua New Guinea where malaria is holoendemic (Burkitt, 1962b, Pope et al., 1967). These tumours normally present in the jaw but can also arise in the orbit, central nervous system, ovaries and abdomen (Magrath, 1990). The endemic form of BL shows the strongest and most consistent association with EBV. In virtually, every tumour analyzed to date, all the malignant cells are EBV genome-positive and each tumour carries a monoclonal EBV episome (Magrath, 1990). The association between EBV and sporadic BL is less consistent. Sporadic BL is histologically identical to eBL occurs at a lower incidence

worldwide and in the west, only 15-20% of these tumours are EBV-positive (Magrath, 1990). A third form of BL has been recognised in human immunodeficiency virus -positive individuals (Carbone et al., 1998, Gaidano et al., 1998). AIDS-BL is identical histologically and has similar sites of presentation to the sporadic tumour but occurrs predominantly in adults and only around 30% of AIDS-BLs are EBV-positive.

Irrespective of endemic/sporadic/AIDS classification and irrespective of EBV genome status, all BL presents with one of three reciprocal translocations involving chromosome 8 at the c-myc locus at 8q24 and either the immunoglobulin heavy (IgH) chain locus on chromosome 14 (termed the "common" translocation seen in up to 80% tumours) or one of the light chain loci on chromosomes 2 or 22 (termed the "variant" translocations) (Dalla-Favera et al., 1982, Magrath, 1990, Taub et al., 1982). Translocations of the c-myc gene release the gene from its usual regular controls and bring it under the control of the long-range enhancer within Ig loci. This translocation likely occurs as an accident of Ig gene rearrangement during class switching in the germinal centre B cells (Goossens et al., 1998), and leads to unusually high and deregulated expression of c-myc protein. Myc proteins are transcription factors that regulate cell growth, differentiation and apoptosis. Deregulated c-myc expression very likely contributes to the high rate of cell proliferation seen in BL tumours. To address the question whether c-myc activation might abrogate the requirement of EBNA2 and LMP function (both are essential for transformation of primary B cells by EBV *in vitro*), Polack and colleagues introduced an activated c-myc gene with a conditional EBNA2 gene in the virus genome in an LCL cell and showed that overexpression of c-myc is able to induce continuous B cell proliferation independently of EBNA2 and LMP1 and

give cells a BL phenotype (Polack et al., 1996). In addition to the translocations of the c-myc locus, BL cells often show other genetic alterations which may contribute to BL pathogenesis (Lindstrom and Wiman, 2002). The majority of BL cell lines and 30% of BL tumours contain mutations within p53, often within the core domain, which affect the transcription activation function of p53 (Farrell et al., 1991, Gaidano et al., 1991, Vousden et al., 1993, Wiman et al., 1991).

The ability to grow BL cell lines derived from biopsy material *in vitro* has provided useful tools for the study of EBV latency. *In vitro* most BL cell lines retain the original biopsy cell phenotype and grow as single cells expressing EBNA1 only (Latency I, see later). These BL cell lines have a completely different growth pattern and cellular phenotype to Latency III lymphoblastoid cell lines (LCL), in particular they lack expression of activation, adhesion markers (Gregory et al., 1988, Gregory et al., 1987) and many components of the HLA class I antigen processing pathway, which *in vivo* is used as a strategy to escape immune detection by CD8+ T cells (Frisan et al., 1996, Khanna et al., 1994, Masucci, 1990). However, in later passage, some latency I lines spontaneously switch to a Latency III form of infection with full latent protein expression and the cells begin to grow in clumps as a result of expression of activation and adhesion marker (Gregory et al., 1990, Rowe et al., 1987).

Latency I is a more restricted from of latency. As shown in Figure 1.4, in Latency I infected cells, Cp, Wp and the LMPs promoters remain silent and there is activation of an alternative promoter, Qp, located downstream in the BamHI Q fragment (Nonkwelo et al.,

1996, Schaefer et al., 1995b). Qp-initiated transcription leads to the production of the a Q-U-K spliced transcript encoding only EBNA1. In addition, the non-coding EBER RNAs and BARTs are also detectable in Latency I (Brooks et al., 1993, Rowe et al., 1987).

Recently, a second form of restricted virus latency has been identified in a subset (~15%) of eBL in Africa (Kelly et al., 2002). In these BLs, Wp is the dominant promoter, Qp is silent and EBNA1, 3A, 3B, 3C and LP are all expressed in the absence of EBNA2 or the LMPs in a form of Latency termed Wp-restricted Latency (see Figure 1.4) The EBNA2 gene and the unique exons of EBNA-LP have been deleted in these cells and the absence of EBNA2 is thought to be the key determinant of the inactivity of Cp and LMP promoters.

The mechanism of how EBV contributes to BL pathogenesis is still not fully understood. EBV may initially expand the population of cells at risk for translocation or increase the survival or proliferation of a translocation-positive clone. The most popular theory at present is that EBV may contribute to BL development by protecting the cells from apoptosis. EBV-positive and negative subclones of EBV-positive BL cell lines can be generated *in vitro* and used as a useful tool to study the influence of EBV on BL cell growth. In particular, the EBV-negative subclones can be reinfected with the virus *in vitro* (Shimizu et al., 1994) It was found that the presence of EBV is associated with improved growth in low serum, anchorage-independent growth in soft agar and tumourigenicity in nude mice (Shimizu et al., 1994). EBV-positive clones were also significantly more resistant to IFN-α-induced apoptosis (Nanbo et al., 2002). It was reported that transfecting

EBERs into the BL cell lines can confer apoptosis resistance (Nanbo et al., 2002, Takada and Nanbo, 2001).

1.6.3 Hodgkin lymphoma (HL) (Latency II)

Hodgkin lymphoma is a tumour with a malignant population of mononuclear Hodgkin and multinuclear Reed–Sternberg (H-RS) cells greatly outnumbered by a reactive non-malignant cell infiltrate. Classical HL can be divided into four subtypes: nodular sclerosis (ns), mixed cellularity (mc), and rarer forms called lymphocyte-rich (lr) and lymphocyte-depleted (ld). HL is seen worldwide and there are differences in age and subtype distribution. In Europe and the United States, HL shows a high peak in young adults dominated by ns-HL and mc-HL while ns-HL contributes to the lower disease incidence seen at other ages. Monoclonal EBV has been detected in H-RS cells and has been subsequently estimated to occur in 60-80% of mc and ld HL types, 20-40% of ns HL types, a fraction of lr HL cases but only rarely if ever in the pathogenetically distinct ld HL (Anagnostopoulos et al., 2000, Anagnostopoulos et al., 1989, Boiocchi et al., 1993, Weiss et al., 1989, Weiss et al., 1987).

EBV present in H-RS cells consistently displays a Latency II pattern of gene expression (Deacon et al., 1993, Grasser et al., 1994, Herbst et al., 1991, Niedobitek et al., 1997b, Pallesen et al., 1991). As shown in Figure 1.4, in Latency II infected cells, Cp and Wp are silent, EBNA1 transcripts initiate from Qp, LMP1 and LMP2 transcripts initiate from their own promoters and in addition, the EBERs and BARTs are also transcribed (Brooks et al., 1992, Brooks et al., 1993, Deacon et al., 1993, Kerr et al., 1992). Elevated levels of LMP1

and LMP2 suggest a significant pathogenic role for these proteins in the development of EBV positive HL. Studies show that LMP2A can mimic signalling through the BCR complex, leading to activation of the downstream PI3 Kinase /AKT and extracellular signal-regulated kinase (ERK) pathways (Cahir-McFarland et al., 2004). Therefore, LMP2A expression could promote survival of even surface Ig-negative GC cells by allowing them to bypass the requirement of high affinity antigen binding for selection into memory (Kuppers, 2002, Kuppers, 2003) and LMP1 signalling can in turn bypass the need for CD40 ligand mediated T cell help. Thus, EBV infection may play a critical role early in the pathogenesis of HL by allowing crippled GC cells to survive. In addition, it is possible that LMP1 and LMP2 proteins act to prevent apoptosis of crippled HL progenitor GC cells by mimicking cellular activation signals, delivered by CD40 and BCR pathways respectively, that are normally required to rescue GC cells into memory.

The contribution of EBV to the pathogenesis of classical HL is yet to be fully understood. EBV is monoclonal in HR-S cells, implying that HL arises from a single EBV-positive precursor (Anagnostopoulos et al., 1989). One recent report about EBV's role in HL pathogenesis showed that EBV infection of Hodgkin lymphoma cells results in the induction of autotaxin, a secreted tumour-associated factor with lysophospholipase-D activity. Up-regulation of autotaxin increased the generation of lysophosphatidic acid (Skandalos et al.) and led to the enhanced growth and survival of Hodgkin lymphoma cells, whereas specific down-regulation of autotaxin decreased LPA levels and reduced cell growth and viability (Baumforth et al., 2005).

1.6.4 Other EBV-associated malignancies

Nasopharyngeal carcinoma (Latency I/II)

Nasopharyngeal carcinoma (NPC) is a tumour of epithelial origin that occurs with extremely high incidence in China and Southeast Asia, up to 100 times higher than in Western countries. In all these high incidence areas, most tumours preferentially affect males (male-to-female ratio 2-3:1). 100% of poorly/non-differentiated NPC tumours are EBV-positive (Andersson-Anvret et al., 1977). Detection of EBV DNA within the NPC tumour cells themselves was the first evidence that the virus could infect epithelial cells (Wolf et al., 1973, zur Hausen et al., 1970). Terminal repeat analysis showed that EBV is monoclonal in the NPC cells (Raab-Traub and Flynn, 1986).

EBV in NPC displays a Latency I/II form of infection, intermediate between those displayed by BL (Latency I) and by HL (Latency II). EBV gene expression in NPC is generally limited to EBNA1 (from Qp), variable levels of LMP1 and LMP2 and EBER RNAs are also transcribed (Raab-Traub, 2002). In epithelial cells, LMP1 is expressed from a different promoter to that in B cells; this promoter is not dependent on EBNA2 and may therefore allow LMP1 expression in the absence of EBNA2 (Sadler and Raab-Traub, 1995a).

The LMP1 mRNA is usually difficult to amplify from the tumour biopsies and the LMP1 protein is only detectable in a minority (35%) of Chinese NPC samples. By contrast, LMP2A RNA is easy to amplify and LMP2A protein can be detected in approximately 50% of tumours by histochemical staining (Heussinger et al., 2004). Due to the limited

sensitivity of the available antibodies for detecting LMP2, the expression of LMP2 may be underestimated. The fact that patients with NPC reproducibly show serum antibody response against LMP2A or B suggests that the protein is regularly expressed in tumour cells. From an understanding of its many effects, LMP1 is inferred to be the key viral protein in NPC pathogenesis. For example, in cultured epithelial cells, LMP1 induces epidermal growth factor receptor and matrix metalloproteinase 9, and downregulates E-cadherin, all of which may contribute to metastasis and tumour invasion *in vivo* (Fahraeus et al., 1992, Miller et al., 1995, Takeshita et al., 1999).

Gastric carcinoma

EBV is detected in the tissue of about 10% of gastric carcinoma cases throughout the world. In each case, 100% of carcinoma cells are infected with EBV (Shibata and Weiss, 1992). The EBV-positive gastric tumours express EBER, the Qp-initiated EBNA1 mRNA, BARTs and in about 50% cases, the LMP2 transcript, but never LMP1 (zur Hausen et al., 2004). In rare cases it has been possible to immortalize primary gastric epithelial cells by EBV infection and a similar form of latency is established in these cells as in EBV positive tumours which show accelerated malignant properties (Nishikawa et al., 1999, Yoshiyama et al., 1997). This suggests that EBV contributes to the maintenance of the malignant phenotype of EBV positive gastric carcinoma.

T/NK lymphomas

In rare cases, EBV can access the non-B-lymphocyte compartment *in vivo* and the infection carries a significant oncogenic risk. This happens in the case of T/NK cell lymphomas. Two

groups of T/NK lymphomas with different clinical presentations have been recognized. Nasal lymphoma of T or NK cells is a specific extra-nodal lymphoma which presents in the nasal cavity. The other group of T/NK lymphomas are peripheral T-cell lymphomas and most frequently following acute primary EBV infection manifest as virus-associated haemophagocytic syndrome (VAHS) or in the setting of chronic active EBV infection with VAHS-like symptoms. EBV infections of T and NK cells lead to latency II infections and not the fully growth transforming program used by the virus in B cells. However detectable levels of LMP1 protein are restricted to a few tumour cells (Minarovits et al., 1994).

1.7 EBV lytic infection studied *in vitro*

Given the link between epithelial cells and EBV replication *in vivo*, there have been efforts to establish *in vitro* replication models in epithelial cell cultures. However, *in vitro* infection of primary epithelial and T cells with free virus is very inefficient because these CD21-negative cells are largely resistant to infection *in vitro* and infection does not result in transformation (Fingeroth et al., 1999, Imai et al., 1998, Shapiro et al., 1982, Sixbey et al., 1983, Yoshiyama et al., 1997). However, recent work with tonsillar epithelial cultures, where there is spontaneous differentiation, shows that productive EBV infection can be achieved experimentally in at least some cells, but this has not yet been studied rigorously. Alternatively, Shannon-Lowe and her colleagues developed a system where EBV is efficiently delivered into epithelial cells by a process involving transfer from the surface of virus-loaded resting B cells (Shannon-Lowe et al., 2006). In contrast to B-cell infection, high levels of EBERs and LMP1 and 2A transcripts were detected in the first 48 hour post-

infection. Furthermore, EBNA1 was exclusively expressed from Qp-derived mRNA and Wp and Cp remained silent at all time points after infection (Shannon-Lowe et al., 2009).

In vitro most studies of lytic cycle have used B cell lines (BL or LCLs), which either spontaneously enter lytic cycle in a small percentage of cells (~5%) or where to varying extents, lytic cycle can be induced by treatment with phorbol ester, calcium ionophore, 5′azacytidine or sodium butyrate or by vector-mediated BZLF1 expression or by sIg cross-linking (Ben-Sasson and Klein, 1981, Saemundsen et al., 1980, Hudewentz et al., 1980, zur Hausen et al., 1979, Luka et al., 1979, Davies et al., 1991, Baumann et al., 1998, Qualtiere and Pearson, 1980, Ragona et al., 1980). In the best characterised system involving anti-Ig induction of the Latency I Akata-BL cell line, EBV gene expression follows a sequential order and completion of the EBV lytic cycle takes 48-72 hours (Takada and Ono, 1989).

Lytic cycle is initiated by expression of two immediate early genes, BZLF1 (also known as Zta or Zebra protein) and BRLF1, which then transactivate the early genes including BALF2, BHRF1 and BMRF1 leading to viral DNA synthesis. Late viral genes including BLLF1 (encodes for envelope glycoprotein gp350/220), BCRF1 (viral homologue of interleukin-10) and BALF4 (encodes for viral gp110) are then expressed and lead to synthesis of the viral structure proteins. Production of new virus starts with synthesis of viral DNA and packaging into capsids in the host cell nucleus followed by budding through the nuclear membrane resulting in cytoplasmic vesicles containing enveloped virus. These vesicles then fuse with the plasma membrane of the host cell to release the new virus

particles by exocytosis (Rickinson and Kieff, 2007). This process also leads to death of the host cell.

1.8 EBV latent infection studies *in vitro*

Virus binding and entry

The ability of EBV to infect target B cells efficiently is determined by the combined interaction of the abundant viral glycoprotein gp350/220 (Peng-Pilon et al., 1995) with the receptor CD21 on the B cells (Fingeroth et al., 1984, Nemerow et al., 1985) and the viral envelope protein complex gp42/gH/gL with major histocompatability (MHC) class II molecules on the B cells (Li et al., 1997). Both interactions are known to be required for efficient B cell infection and are dispensable for the much less efficient entry of EBV into other cell types, such as epithelial cells (Borza and Hutt-Fletcher, 2002, Li et al., 1995). EBV enters B cells by endocytosis, involving fusion of the viral envelope with the host cell plasma membrane and this is triggered by cross-linking of CD21 molecules (Nemerow and Cooper, 1984, Tanner et al., 1987). EBV genome delivery to the nucleus is a key rate-limiting step to B cell transformation. For EBV infection of resting B cells, only 10-15 % of total bound viruses deliver their genomes to the cell nucleus and importantly, a single viral genome in the nucleus is capable of driving B cell proliferation (Henderson et al., 1977, Hurley and Thorley-Lawson, 1988, Shannon-Lowe et al., 2005, Sugden and Mark, 1977).

The EBV genome, which is linear in the virion, circularises soon after virus entry to produce the covalently closed extrachromosomal episome (Adams and Lindahl, 1975,

Alfieri et al., 1991, Hurley and Thorley-Lawson, 1988). This circularisation takes place within 12 to 16 hours of infection. Episomes are maintained by the host cell DNA polymerase (Adams, 1987) and amplification of the EBV genome relative to the cell genome occurs after transformation, such that most latently infected cells harbour numerous episomes (Sugden et al., 1979).

Viral gene transcription

After the initial events of viral infection involving viral binding by adsorption to CD21, internalisation of the viral episome within the infected cell nucleus, the BamHI W promoter, Wp, present in each BamHI W repeat of the EBV genome, is selectively activated immediately post-infection, within 12 to 16 hours (Alfieri et al., 1991, Allday et al., 1989, Moss et al., 1981, Rooney et al., 1992). This activation leads to selective expression of EBNA-LP and EBNA2, detectable within 12-24 hours post-infection. EBNA-LP is hyperexpressed at this time whereas EBNA2 gradually rises to LCL-like levels. Differential splicing of Wp-initiated mRNAs leads to the expression of both EBNA-LP and EBNA 2 (Rogers et al., 1990, Wang et al., 1987b).

Figure 1.5, adapted from (Qu and Rowe, 1992), shows a schematic of the splicing patterns of Wp- and Cp-initiated transcripts. The N-terminus of EBNA-LP is encoded by multiple W_1 and W_2 exons within the *Bam*HI W repeats, while the C-terminus is encoded by two unique *Bam*HI Y exons, Y_1 and Y_2. As the W repeat exons are located within the 5′ untranslated region (UTR) of all EBNA transcripts, alternative splicing of the first 40nt

exon W_0 to an alternative splice acceptor W_1' 5bp into the 66nt W_1 exon is required to create a translation initiation codon specific for the production of EBNA-LP. These Wp-transcripts splice into the next downstream 132nt W_2 exon and then through multiple repeats of W_1 and W_2 exons and eventually into the Y_1 and Y_2 exons.

EBNA2 is encoded further downstream in the BYFR1 ORF spanning across the *Bam*HI Y and *Bam*HI H fragment junctions. Wp-initiated transcripts productive for EBNA2 splice from W_0 to W_1, thereby ablating the translation initiation codon for EBNA-LP, then into W_2, through multiple W_1 and W_2 exons, and finally into the Y1Y2Y3H (Alfieri et al., 1991, Sample et al., 1986, Speck and Strominger, 1985).

EBNA-LP and EBNA2 reach the level that is maintained in transformed B cells (LCL) by 24 to 32 hours after infection (Alfieri et al., 1994). EBNA2, either alone or acting together with EBNA-LP, activates the alternative promoter Cp, detectable 48-72 hours post-infection (Alfieri et al., 1991, Jin and Speck, 1992, Sung et al., 1991). Cp then becomes the dominant promoter and Wp-initiated transcripts begin to wane (Woisetschlaeger et al., 1991, Woisetschlaeger et al., 1990). The Cp-initiated transcripts, in which the C_1 and C_2 exons replace the W_0 exon, are highly spliced to generate mRNAs and encoded all six EBNAs. Some of these Cp-initiated transcripts exploit another splice donor upstream of EBNA2 to splice to the downstream U exon and then into the EBNA3 and EBNA1 encoding exons. An internal ribosome entry site has recently been identified in the U exon which would permit efficient translation of these downstream EBNAs (Isaksson et al.,

2003). Thus by alternative RNA splicing, the Cp-initiated transcripts are capable of encoding EBNAs 1, 2, -LP, 3A, 3B, and 3C (Bodescot and Perricaudet, 1986, Sample et al., 1986, Speck and Strominger, 1985). EBNA 3A, 3B, 3C and 1 proteins are detectable 20-32 hours after infection and reach LCL levels after 48-72 hours. In addition to EBNA2, it has also been reported that EBNA1 can also increase Cp and LMP1 promoter activity by binding to the oriP enhancer and thereby increasing transcription of the latent antigens (Reisman and Sugden, 1986). By contrast, EBNA3 may limit EBNAs transcription by competing with EBNA2 for binding to RBP-Jκ, the anchor for EBNA2 on the Cp enhancer to inhibit EBNA2 mediated transactivation (Johannsen et al., 1996, Waltzer et al., 1996).

Subsequently, EBNA2 and EBNA-LP activate the LMP promoters (LMP1, 2A and 2B) 72-96 hours post-infection (Abbot et al., 1990, Sung et al., 1991, Wang et al., 1990b, Zimber-Strobl et al., 1991). The most abundant EBV-encoded RNAs (the EBERs) the BamHI A rightward transcripts are also expressed following these early events (Alfieri et al., 1991, Chen et al., 2005b).

Expression of the full range of Latency III viral proteins triggers cells to enter the cell cycle and proliferate. EBNA2, either alone or acting with EBNA-LP, exerts many effects on cellular and viral gene expression leading to a G0 to G1 cell cycle transition as marked by the induction of cyclin D2 (Sinclair et al., 1994). Among EBV upregulated cell genes, c-myc and CD21 RNA and c-myc protein expression are up at 16 to 24 hours after infection (Alfieri et al., 1991). Accompanying this is a change in cellular phenotype from a resting B cell to a proliferating LCL; cells begin to enlarge and clump together in culture, expressing

adhesion molecules and activation markers (Hurley and Thorley-Lawson, 1988). By 48 hours after infection, 15% of cells are in S phase and at 72 hours a substantial fraction of infected cells have divided (Rickinson and Kieff, 2007).

1.9 Regulation of latent promoters involved in Latency I, II, and III

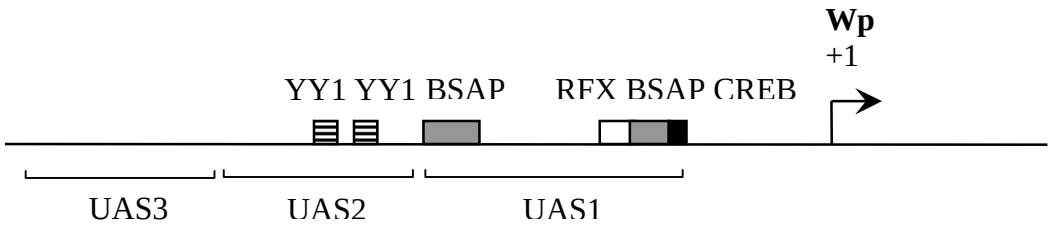
1.9.1 Wp

The BamHI W promoter, Wp is present in each BamHI W repeat of the EBV genome and is selectively activated within 12-16 hours post-infection (Alfieri et al., 1991, Allday et al., 1989, Moss et al., 1981, Rooney et al., 1992). Wp may be designed to function particularly well in the quiescent environment of a resting B cell due to its presence in multiple copies (Woisetschlaeger et al., 1990). The ladder of EBNA-LP species seen in the early stages of EBV infection supports the notion that transcription initiates from multiple copies of Wp (Finke et al., 1987, Rooney et al., 1989).

The factors that determine Wp activation in B cells are poorly understood. It was initially suggested that EBV binding to its cellular receptor, CD21 on B cells triggers an NF-κB-dependent intracellular pathway and mediates transcriptional activation of Wp (Sugano et al., 1997). As shown in Figure 1.6, there are three positive regulatory regions or upstream activating sequences (UAS) located upstream of Wp, UAS1, UAS2 and UAS3 (Bell et al., 1998, Ricksten et al., 1988). UAS2 and UAS3 were found to be lineage-independent in their activity, while UAS1 activity is preferentially in B cells (Bell et al., 1998). UAS2 contains two binding sites for the ubiquitous transcription factor Yin-Yang 1 (YY1) and shows lineage-independent activity in reporter assays. By contrast UAS1 contains two

binding sites for the B cell-specific activator proteins (BSAP) protein, plus sites for the ubiquitous regulatory factor X (RFX) and the cAMP response element (CRE)-binding protein (CREB), and has B cell-specific activity (Bell et al., 1998, Kirby et al., 2000, Tierney et al., 2000a) (Figure 1.6).

Figure 1.6 Schematic diagram of Wp promoter and transcription factor binding sites



Experiments using recombinant EBVs carrying two tandemly-arranged copies of Wp deleted either for UAS1 and/or UAS2 showed that UAS2 was completely dispensable for Wp activation and subsequent B cell transformation, whereas UAS1 was absolutely essential for both activities (Tierney et al., 2007). This was in agreement with earlier studies which showed that deletion of -369 to -169bp upstream of Wp, a region overlapping UAS1, in a recombinant virus, was unfavourable for B-cell transformation (Yoo et al., 2002). In addition, recombinant virus carrying two Wp copies in which both BSAP sites are inactivated, like the UAS1 knockout, showed greatly reduced Wp activity in resting B cells and did not transform B cells. It inferred that EBV ensures the B-cell specificity of its growth-transforming function by exploiting BSAP/Pax5 as a lineage-specific activator of the transforming programme (Tierney et al., 2007).

1.9.2 Cp

As shown in Figure 1.7, the upstream regulatory regions of Cp are important for promoter activity and both viral and cellular proteins have been implicated in Cp regulation. In reporter assays, EBNA1 binding to oriP can activate transcription from Cp; multiple EBNA1 homodimers bind to the EBNA1-dependent enhancer within FR of oriP, and activate Cp several kilobases downstream (Zetterberg et al., 2004, Nilsson et al., 1993, Puglielli et al., 1996, Reisman and Sugden, 1986, Sugden and Warren, 1989). EBNA2 is also a transcriptional activator of Cp. The EBNA2-response element in Cp contains binding sites for the cellular transcription factors CBF1 (Cp binding factor 1)/RBP-Jκ and CBF2 both of which are required for EBNA2-mediated activation of Cp in reporter assays (Fuentes-Panana and Ling, 1998, Jin and Speck, 1992, Ling et al., 1993, Henkel et al., 1994, Waltzer et al., 1994, Fuentes-Panana et al., 2000, Grossman et al., 1994). CBF2 was identified as the AUF1/hnRNP D protein which is regulated by the cyclic AMP-protein kinase A (cAMP/PKA) signalling pathway, and this pathway contributes to the regulation of Cp activity (Fuentes-Panana et al., 2000).

The Cp promoter is also regulated by the EBNA3 proteins, which can obstruct EBNA2-mediated transactivation of Cp by preferentially binding to RBP-Jκ (Robertson et al., 1995). Also important for Cp activity are a CCAAT box which binds nuclear factor Y (NF-Y), promoter-proximal GC-rich elements that interact with Sp1, and a C/EBP-binding site (Borestrom et al., 2003, Nilsson et al., 2001, Puglielli et al., 1996).

Figure 1.7 Schematic diagram of Cp promoter and transcription factor binding sites

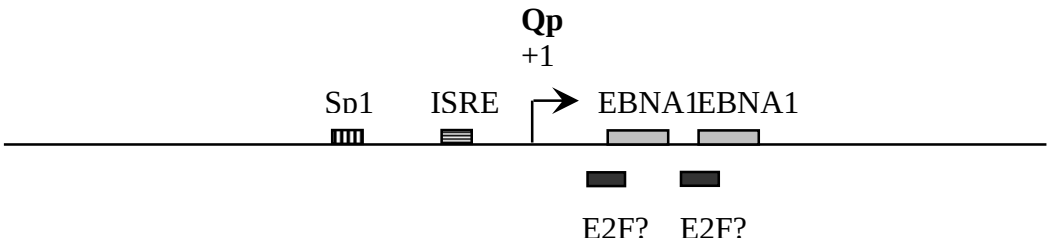
Although Cp is the promoter of choice in most LCLs, it is in fact dispensable for B cell immortalisation *in vitro*. Recombinant virus with a deletion of the key Cp regulatory regions (including the GRE and EBNA2 response element) and the C1 and C2 exons transformed B cells with wild-type efficiency (Swaminathan, 1996). Wp was instead adopted as the EBNA promoter and appeared to compensate entirely for the lack of Cp. Deletion of a 200bp section, comprising the entire EBNA2-response element, generated LCLs with substantially diminished Cp activity, but in which the immortalising capacity of the virus remained intact (Yoo and Speck, 2000, Yoo et al., 1997).

1.9.3 Qp

Qp located in the BamHI Q fragment is an alternative EBNA1 promoter that allows selective EBNA1 expression in the absence of the other EBNAs. Unlike the Cp and LMP1 promoters, it is not dependent on EBNA2 for activity. Qp is a TATAA-less promoter whose architecture is similar to the promoters of housekeeping genes and it is active in a wide range of cell types, including non-lymphoid cells (Schaefer et al., 1995a). As shown in Figure 1.8, the key elements for Qp regulation are clustered in an 80bp sequence around the transcription start site (Schaefer et al., 1991). Two binding sites for E2F, which overlap

the EBNA1 binding sites were identified *in vitro* studies (Sung et al., 1994), but *in vivo* footprinting failed to confirm this (Salamon et al., 2001).

Figure 1.8 Schematic diagram of Qp promoter and transcription factor binding sites



Qp is positively regulated by interferon regulatory factors (IRFs) which associate with an interferon stimulated response element (ISRE) just upstream of the transcriptional start site (Nonkwelo et al., 1997, Schaefer et al., 1997, Zhang and Pagano, 1997). IRF2 binding to Qp activates the promoter and is the principal factor bound when Qp is active in Latency I BL lines (Schaefer et al., 1997). Two STAT binding sites adjacent to the ISRE have been described and STAT proteins have been shown to activate Qp (Chen et al., 1999a). A Sp1-like site, which may bind Sp1 also identified by *in vivo* footprinting upstream of the Qp transcription start site (Salamon et al., 2001). EBNA1 expression from Qp is autoregulated by binding of EBNA1 to two low-affinity sites just downstream of the Qp start site, which inhibits transcription initiation (Sample et al., 1992, Schaefer et al., 1997).

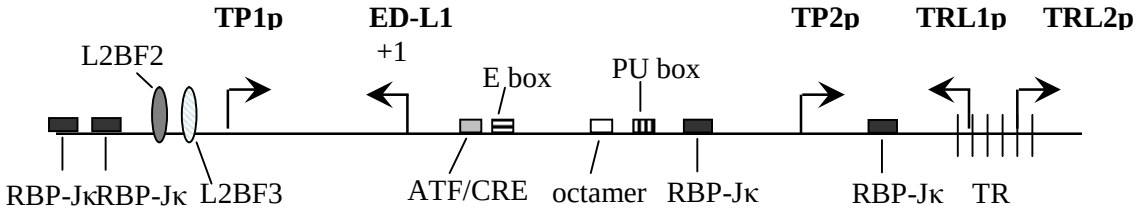
1.9.4 LMP1 promoters

The LMP1 promoter (ED-L1) is responsive to transactivation by EBNA2 via binding to RBP-Jκ (Fahraeus et al., 1990). As shown in Figure 1.9, there are two binding sites for RBP-Jκ in the LMP1 transcription regulatory sequence (LRS) but only the promoter proximal site is involved in transcriptional activation (Johannsen et al., 1995, Laux et al., 1994b). The cAMP response element (CRE) in the ED-L1 promoter has been shown to be one of the critical sites for LMP1 transactivation. Both EBNA2-dependent and -independent transactivation of LMP1 require an intact CRE in LRS upstream of the transcription initiation site of ED-L1 promoter. Mutations in this site lead to a drastic decrease in promoter activity in reporter assays (Fahraeus et al., 1994). Downstream of the RBP-Jκ site, there is an E-box which may bind a repressor complex (Sjoblom-Hallen et al., 1999), an octamer motif which binds a POU-domain protein (Sjoblom et al., 1995) and a purine-rich PU box sequence which binds PU.1/Spi-1 and Spi-B (Johannsen et al., 1995, Laux et al., 1994a, Sjoblom et al., 1995). PU.1 is a B cell-specific factor which has a role in EBNA2 activation of LMP1p as well as that of RBP-Jκ (Johannsen et al., 1995, Sjoblom et al., 1995). In different experimental situations, reportedly ED-L1 promoter can also be activated by EBNA1 binding to oriP (Gahn and Sugden, 1995), by EBNA3C expression (Allday et al., 1993) and by signals from the protein kinase A and C pathways (Fahraeus et al., 1994, Rowe et al., 1992).

In epithelial cells, on the other hand, where EBNA2 is not expressed, transcription of LMP1 mRNA can also initiate from TRL1p, a TATA-less promoter situated in the first terminal repeat (Gilligan et al., 1990, Sadler and Raab-Traub, 1995a). TRL1p contains two

GC boxes (Sadler and Raab-Traub, 1995a), I and II and by binding to the GC boxes, the cellular Sp family proteins including Sp1 and Sp3 function as transcriptional activators and regulate this promoter (Tsai et al., 1999).

Figure 1.9 Schematic diagram of LMP promoters and transcription factor binding sites



1.9.5 LMP2 promoters

The LMP-2A promoter is located approximately 3.2 kb downstream (leftward) of the LMP-1 transcription start site. As shown in Figure 1.9, the regulatory sequence of LMP2A promoter, TP1p, contains two RBP-Jκ binding sites, and *in vivo* footprinting revealed binding of RBP-Jκ to its recognition sequence in B cell lines actively using TP1p, whereas no typical RBP-Jκ footprints could be observed in cells where TP1p was silent (Salamon et al., 2003). In addition to the two RBP-Jκ binding sites, two other sites (L2BF2 and L2BF3) were also found in the promoter proximal to the RBP-Jκ binding sites (Hofelmayr et al., 1999). LMP2B is expressed from a promoter, termed TP2p in the same bidirectional

promoter region as LMP1. Details are uncertain about specific factors controlling LMP2B expression.

1.10 Latent gene products

1.10.1 EBNA-LP

EBNA-leader protein (EBNA-LP or EBNA 5), alongside EBNA 2, is one of the first EBV proteins expressed during B cell infection (Alfieri et al., 1991). EBNA-LP is encoded by the repeating W1 and W2 exons in the BamHI W fragments and the unique Y1 and Y2 exons in the BamHI Y fragment (Bodescot et al., 1984, Dillner et al., 1986, Sample et al., 1986, Speck et al., 1986), which generates a protein with a 66 amino acid repeat region and a unique 45 amino acid carboxy terminus. The size of the protein varies between 20-130kd in virus isolates, depending on the number of W1 and W2 repeats present and is seen on western blotting as a ladder of bands at 6-8kd intervals (Dillner et al., 1986, Finke et al., 1987, Wang et al., 1987b).

EBNA-LP functions to co-activate EBNA2-dependent activity of the EBNA2-responsive promoters, Cp and LMP1 (Harada and Kieff, 1997, Nitsche et al., 1997). While genetic analyses have mapped this co-activation function to the repeat region of the protein, the unique C-terminal domain appears to modulate EBNA-LP activity (Harada and Kieff, 1997, Peng et al., 2000b, Kitay and Rowe, 1996, McCann et al., 2001). EBNA-LP and EBNA 2 together induce a G0 to G1 transition as measured by upregulation of cyclin D2 mRNA (Sinclair et al., 1994).

EBNA-LP is phosphorylated on serine residues at least once in each W2 repeat *in vivo* during the late G2 stage of the cell cycle (Kitay and Rowe, 1996, Petti et al., 1990). It has been shown that casein II kinase, p34cdc2 and DNA-PK are all capable of phosphorylating EBNA-LP *in vitro* (Han et al., 2001, Yokoyama et al., 2001). Phosphorylation of serine residue 36 in W_2 is thought to be essential for EBNA-LP function (McCann et al., 2001, Peng et al., 2000b). EBNA-LP is highly associated with the nuclear matrix fraction. By immune microscopy, EBNA-LP has been detected in ND10 bodies and some spread throughout the nucleus (Wang et al., 1987b, Petti et al., 1990, Szekely et al., 1996).

Most studies of EBNA-LP using recombinant viruses focused on the carboxyl-terminal two exons because mutations in the repeating exons are more difficult to construct. EBV recombinants expressing a truncated EBNA-LP lacking the unique C-terminus have impaired transforming capabilities and the resulting LCLs grow slowly, showing that the unique region is important, but not essential for transformation (Hammerschmidt and Sugden, 1989, Mannick et al., 1991). Recombinant virus carrying only two copies of BamHI W fragments, producing EBNA-LP with only two repeat regions have impaired transforming capabilities (Tierney et al., 2007). Therefore, the length of the EBNA-LP determined by the number of repeat regions may be important.

Mannick et al immunoprecipated EBNA-LP from cell extracts, and found that EBNA-LP associated with members of the heat shock protein 70 family (HSP73 and HSP72) (Mannick et al., 1995), which colocalised with EBNA-LP in ND10 bodies (Szekely et al., 1995). Recently, it has been shown that overexpressing HSP 72 up-regulates EBNA-LP

coactivation with EBNA2 (Peng et al., 2007). Sp100 has also been implicated in EBNA-LP coactivation, whereas HA95 and PKA are implicated in negative regulation of EBNA2 -and EBNA-LP- mediated transcription (Han et al., 2002, Ling et al., 2005).

1.10.2 EBNA2

EBNA2 is one of the first viral proteins to be expressed after EBV infection of B cells *in vitro* (Alfieri et al., 1991, Woisetschlaeger et al., 1990, Woisetschlaeger et al., 1991) and is essential for the B cell immortalising ability of EBV (Hammerschmidt and Sugden, 1989, Skare et al., 1985) as exemplified by the non-transforming phenotype of the EBNA2-deleted virus P3HR1 (Miller et al., 1974, Menezes et al., 1975, Bornkamm et al., 1982, Rabson et al., 1982). Reintroduction of EBNA2 into P3HR1 cells restored the transformation capacity of the virus (Hammerschmidt and Sugden, 1989). Experiments using conditional EBNA2 also showed that EBNA2 is not only essential for initiation of, but also for maintenance of B cell immortalization (Kempkes et al., 1995a).

The essential role of EBNA2 in B cell transformation is due to its ability to act as a potent transcriptional activator (Cohen and Kieff, 1991), upregulating transcription of several viral and cellular genes containing EBNA2-response elements in their promoters. Viral targets include the EBNA promoter, Cp (Jin and Speck, 1992, Sung et al., 1991) and the LMP1 and LMP2A promoters (Figure 1.4) (Abbot et al., 1990, Fahraeus et al., 1990, Ghosh and Kieff, 1990, Zimber-Strobl et al., 1993, Wang et al., 1990b). The importance of EBNA2 in transactivating LMP1 and LMP2 gene expression in B cells is highlighted by the absence of the LMP proteins in BL lines in which the EBNA2 gene has been deleted (Abbot et al.,

1990, Kelly et al., 2002, Zimber-Strobl et al., 1991). In its capacity as a transcriptional activator, EBNA2 has a C-terminal acidic activation domain that recruits transcription factors to promoters, including TFIIB, TAF40, and TFIIH (Cohen and Kieff, 1991, Tong et al., 1995a, Tong et al., 1995b). Interestingly, the EBNA2 transactivation domain closely resembles the herpes simplex virus VP16 acidic domain which also recruits transcription factors (Cohen, 1992). EBNA2 can interact with the histone acetyltransferases (HATs) p300, CBP and PCAF (Wang et al., 2000), and such associations may be central to EBNA2-mediated transactivation.

EBNA2, either alone or acting with EBNA-LP, exerts many effects on cellular and viral gene expression leading to a G0 to G1 cell cycle transition as marked by the induction of cyclin D2 (Sinclair et al., 1994). The mechanism of activation and the range of cellular targets involved are still not fully understood. However it is known that in experimental models EBNA2 up-regulates cellular genes including the proto-oncogene c-myc, B cell activation marker CD23, the viral receptor CD21, chemokine receptor CCR7, and proto-oncogene cfgr (Burgstahler et al., 1995, Cordier et al., 1990, Knutson, 1990, Wang et al., 1991, Wang et al., 1987a). More recently, microarray analysis has identified numerous cellular genes upregulated in LCLs in response to EBNA2 expression (Zhao et al., 2006).

EBNA2 does not bind directly to DNA but interacts with RBP-Jκ, a cellular sequence-specific DNA binding protein that typically acts as a transcriptional repressor (Hsieh and Hayward, 1995, Waltzer et al., 1995); this interaction between EBNA2 and RBP-Jκ is essential for B lymphocyte immortalisation (Zimber-Strobl et al., 1994, Yalamanchili et al.,

1994, Grossman et al., 1994, Ling et al., 1994, Henkel et al., 1994). Recombinant viruses carrying an EBNA2 gene lacking the interaction domain are not able to transform B cells (Yalamanchili et al., 1994). At least one consensus RBP-Jκ binding site is found in almost all the EBNA2-responsive elements in the promoters of EBNA2-transactivated viral and cellular genes. EBNA2 is thought to convert RBP-Jκ from a repressor into an activator to upregulate its target genes in B cells; EBNA2 binding to RBP-Jκ can mask the repression domain of RBP-Jκ and bring the transactivation domain within range of the Cp TATAA box (Hsieh and Hayward, 1995, Kao et al., 1998, Ling et al., 1993). Other elements important for EBNA2-reponsiveness include the PU.1 and CRE binding site in the LMP1 promoter (Johannsen et al., 1995, Sjoblom et al., 1995) and the AUF1 binding sites in the Cp and CD21 promoters (Fuentes-Panana et al., 2000).

In activating transcription through RBP-Jκ, EBNA2 mimics a constitutive Notch receptor. The WWP sequence in EBNA2 is similar to the WFP sequence in the Notch receptor intracellular domain that normally mediates tight association with RBP-Jκ in tissue development (Tamura et al., 1995). Notch binds RBP-Jκ in the nucleus through the same site as EBNA2, and also transactivates genes by interacting with RBP-Jκ and converting it into an activator (Schroeter et al., 1998, Struhl and Adachi, 1998, Hsieh et al., 1996). In BL cells, Notch can transactivate viral EBNA2 target promoters in transient transfection assays (Hofelmayr et al., 1999), and in an LCL Notch can transiently substitute for EBNA2 in maintaining proliferation (Hofelmayr et al., 2001).

1.10.3 BHRF1

EBV encodes two homologues of the cellular anti-apoptotic Bcl2 protein, BALF1 and BHRF1 (Henderson et al., 1993, Horner et al., 1995, Marshall et al., 1999), which are expressed soon after EBV infection of B cells (within 24 hours). Single BHRF1$^-$ or BALF1$^-$ mutants were capable of generating LCLs at high virus doses however, a recombinant virus in which both genes were inactivated was unable to transform infected B cells, which died by apoptosis (Altmann and Hammerschmidt, 2005). These results suggest that BHRF1 and BALF1 can partially compensate for each other. Importantly when pre-activated B blasts were infected with the double knockout clonal LCLs were obtained. These studies indicated that either BALF1 or BHRF1 is essential to establish LCLs but are dispensable for their continued proliferation. Interestingly, it was also suggested that this EBV anti-apoptotic gene could provide a survival advantage to the infected cells favouring their developing into memory B cells *in vivo*.

Recently, it has been reported the BHRF1 gene expression in newly infected B cells is temporally linked to Wp activation and the presence of W/BHRF1-spliced transcripts. Low-level BHRF1 transcripts and protein remain detectable in these cells long-term, independently of any lytic cycle entry (Kelly et al., 2009). Stable transfection of the BHRF1 gene into P3HR-1 cells rescued the cells from the apoptosis induced by dominant negative EBNA1 expression and moreover, knockdown of BHRF1 expression in P3HR-1 cells resulted in increased cell death. These results indicate that EBV is essential for the survival of P3HR-1 cells and that BHRF1 functions as a survival factor (Watanabe et al.,

2010). This work suggested that BHRF1, in the context of Wp-restricted BL, may contribute to virus-associated lymphomagenesis *in vivo.*

1.10.4 EBNA1

EBNA1 is a nuclear protein encoded by the BKRF1 ORF. EBNA1 transcripts are initiated from Cp or Wp in Latency III cells (Y3-U-K spliced transcript) and Qp in Latency I/II cells (Q-U-K spliced transcript). EBNA1 from the B95.8 EBV strain consists of 641 amino acids with an apparent size of 76kd. EBNA1 protein has four components: (a) an Arg-rich amino terminal 89 aa region, (b) a Gly-Ala repeat 239 aa domain, (c) an Arg-rich short 58 aa domain, (d) a long hydrophilic carboxy terminus (Hennessy and Kieff, 1983).

EBNA1 was first identified as a protein that binds to chromosomes during mitosis, and is unique among the EBNAs in this regard (Reedman and Klein, 1973, Ohno et al., 1977). The chromosome-binding functions of EBNA1 are mediated through its interaction with a cis-acting element, termed oriP within the EBV genome (Yates et al., 1984, Yates et al., 1985). OriP has at least two components with binding sites for EBNA1: a family of 20 copies of a 30bp repeat, FR, and a dyad symmetry, DS, consisting of four copies of the 30bp repeat, two in tandem and two in a larger dyad symmetry (Ohno et al., 1977, Petti et al., 1990, Reedman and Klein, 1973). The chromosome-binding function of EBNA1 is essential for EBV episome maintenance, allowing the viral genome to be replicated once per cell cycle by the host cell replication machinery, alongside the cellular genome and allows faithful partitioning to daughter cells (Adams, 1987, Lee et al., 1999, Yates et al., 1985, Harrison et al., 1994, Harris et al., 1985).

Binding of EBNA1 to the FR may also enhance transcription from viral promoters Cp, Wp, LMP1 and LMP2B (Gahn and Sugden, 1995, Puglielli et al., 1996, Reisman and Sugden, 1986, Sugden and Warren, 1989). Conversely EBNA1 binding to two low affinity sites downstream of the Qp promoter mediates negative regulation of Qp and presumably serves to limit EBNA1 expression in Latency I/II infections (Sample et al., 1992, Sung et al., 1994). EBNA1 function may also contribute to the growth transformation properties of EBV. It was reported that EBNA1 is required for survival in BL cells, and inhibition of EBNA1 decreases survival of the cells by inducing apoptosis (Kennedy et al., 2003). In addition, delivery of a dominant-negative EBNA1 into EBV-positive BL cells can inhibit their growth (Nasimuzzaman et al., 2005). Furthermore, experiments using siRNAs against EBNA1 could also inhibit the growth and survival of an EBV-positive cell line (Yin and Flemington, 2006). By using an EBNA1-deficient EBV mutant virus, one group has reported that EBNA1 is not essential to establish LCLs but promotes the efficiency of this process significantly (Humme et al., 2003).

1.10.5 The EBNA3 family

The EBNA3 family consists of three proteins, EBNA3A, -3B and -3C which are large multifunctional proteins of sizes 145kDa, 165kDa and 155 kDa, respectively. The EBNA3 genes are tandemly placed in the genome, and are transcribed from the far-upstream Cp or Wp. Use of recombinant viruses has shown that EBNA3A and 3C are essential for B lymphocyte growth transformation, while EBNA3B is not (Maruo et al., 2003, Maruo et al., 2006, Tomkinson and Kieff, 1992b, Tomkinson et al., 1993). The EBNA3 proteins differ

between type 1 and type2 viruses, however, use of recombinant viruses has shown that type1 and type2 EBNA3 have similar effect on B-lymphocyte growth transformation (Tomkinson and Kieff, 1992a). The EBNA3 proteins are thought to function as transcriptional regulators of viral and cellular genes. Like EBNA2, EBNA3 proteins can interact with RBP-Jκ, and even bind more strongly than EBNA2 does (Johannsen et al., 1996, Robertson et al., 1995, Robertson et al., 1996, Krauer et al., 1996, Zhao et al., 1996). This has led to the idea that the EBNA3s may modulate EBNA2 activation of RBP-Jκ repressed promoters by competing for RBP-Jκ binding (Waltzer et al., 1996). EBNA3A overexpression decreased EBNA2 association with RBP-Jκ, decreased c-myc expression, and caused G0/G1 growth arrest with prolonged viability (Cooper et al., 2003). It has been shown that EBNA3C can repress transcription from Cp through interactions with RBP-Jκ and a histone deacetylase (HDAC) (Bain et al., 1996, Radkov et al., 1997). In addition, EBNA3C is involved in transcriptional activation, and can cooperate with EBNA2 to coactivate the LMP1 promoter (Allday et al., 1993, Marshall and Sample, 1995). This ability to co-activate the LMP1 promoter with EBNA2 occurs independently of RBP-Jκ binding but instead is dependent on a PU.1 site within the promoter (Zhao and Sample, 2000, Lin et al., 2002). EBNA3C has also been shown to co-operate with oncogenic Ras in the immortalisation of primary rodent fibroblasts (Parker et al., 1996).

1.10.6 LMP1

LMP1 is a 66kDa integral membrane protein encoded by the BNLF1 ORF, and consists of a short N-terminal cytoplasmic tail of 17aa residues, six hydrophobic transmembrane segments and a long C-terminal cytoplasmic region of 200aa residues (Fennewald et al.,

1984). In LCLs, LMP1 is principally encoded by a 2.8 kb mRNA that initiates from the LMP1 promoter, ED-L1 and which requires transactivation by EBNA2 (Fennewald et al., 1984, Ghosh and Kieff, 1990, Hudson et al., 1985, Abbot et al., 1990, Wang et al., 1990b, Fahraeus et al., 1990). However in epithelial cells, a different promoter, TRL1P is used to drive LMP1 expression, giving rise to a 3.5 kb LMP1 mRNA and initiating from heterogeneous sites in the first terminal repeat of the EBV genome (Sadler and Raab-Traub, 1995a). This distinct promoter is not dependent on EBNA2, and therefore allows LMP1 expression in epithelial cells such as nasopharyngeal carcinoma cells, in the absence of EBNA2 protein.

LMP1 is generally considered to be an oncogenic protein. In single gene transfer experiments under heterologous promoters, LMP1 has transforming effects in continuous rodent fibroblast cell lines (Baichwal and Sugden, 1988, Moorthy and Thorley-Lawson, 1993, Wang et al., 1985). In Rat-1 cells, expression of LMP1 alters cell morphology, and enables cells to grow in low serum medium and leads to loss of contact inhibition so that cells heap up in monolayer culture (Wang et al., 1985). Expression of LMP1 in the B lymphocytes of transgenic mice predisposes them to develop lymphomas late in life (Kulwichit et al., 1998).

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LMP1 expression also has significant effects on growth of epithelial cells and can inhibit differentiation and cause morphological transformation (Dawson et al., 1990, Fahraeus et al., 1993). LMP1 expression in epithelial cells induces epidermal growth factor receptor (EGFR) expression (Miller et al., 1995). In B and epithelial cells LMP1 can induce anti-

apoptotic factors A20 and Bcl2 (Henderson et al., 1991, Laherty et al., 1992, Rowe et al., 1994). As a mimic of activated CD40, LMP1 can suppress virus reactivation by inhibiting transcription of a viral immediate early gene BZLF1, and may therefore contribute to EBV latency (Adler et al., 2002).

LMP1 expression in EBV-negative BL cells or B cells induces a number of phenotypic changes in the cell, including upregulation of activation markers CD21, CD23, CD30, CD39, CD40 and CD44 and of cell adhesion molecules ICAM1, LFA1 and LFA3 and can even restore antigen processing function to BL cells (Wang et al., 1990a, Peng and Lundgren, 1993, Huen et al., 1995, Rowe et al., 1995). It is thought to do this by mediating signal transduction pathways that lead ultimately to the activation of transcription factors, such as nuclear factor kappaB (NF-κB) and activating protein-1 (AP-1) (Huen et al., 1995, Laherty et al., 1992, Mitchell and Sugden, 1995, Kieser et al., 1997, Eliopoulos and Young, 1998, Hatzivassiliou et al., 1998). The domains of LMP1 required for NF-κB activation are the C-terminal activating regions, CTAR-1 (residues 194-232) and CTAR-2 (residues 351-386) (Huen et al., 1995). LMP1 protein mimics a constitutively active tumour necrosis factor (TNF) receptor, CD40, promoting B cell proliferation by interaction with signalling molecules such as TNF receptor-associated factors (TRAFs) and TNF receptor-associated death domain protein (TRADD) through the CTAR regions (Mosialos et al., 1995, Devergne et al., 1996, Gires et al., 1997, Izumi and Kieff, 1997, Kilger et al., 1998). CTAR1 and CTAR2, play critical roles in the transforming phenotype of LMP1 (Liebowitz et al., 1992, Izumi et al., 1997, Izumi and Kieff, 1997, Kaye et al., 1999). Deletion of CTAR1 showed it to be essential for primary B cell transformation (Kaye et al., 1999,

Izumi et al., 1997); deletion of CTAR2 did not fully abrogate transformation but severely impaired growth of the resulting LCLs (Izumi and Kieff, 1997).

1.10.7 LMP2A and 2B

Two integral membrane proteins, LMP2A and LMP2B are initiated from promoters 3kb apart in the EBV genome and generated by alternative splicing (Sample et al., 1989). Transcription of the LMP2 genes occurs across the TRs and therefore requires circularisation of the viral genome (Kieff and Rickinson, 2007). The first exons of LMP2A and LMP2B are the only unique exons and all other eight exons are the same to both. The 5′ exon of LMP2B is noncoding, whereas the 5′ exon of LMP2A encodes a 119-amino-acid cytoplasmic domain which is implicated in cell signalling (Beaufils et al., 1993). LMP2A has been reported to localise to the same site in the plasma membrane as LMP1 in latently infected B lymphocytes (Longnecker and Kieff, 1990), whereas other studies report a distinct intracytosolic pattern of LMP2 distribution (Lynch et al., 2002).

LMP2 has been extensively mutated in EBV recombinants and the experiments showed that LMP2 does not effect EBV transformation of primary B cells into LCLs or LCL survival (Rochford et al., 1997, Kim and Yates, 1993, Longnecker et al., 1993, Longnecker et al., 1992, Speck et al., 1999).

1.10.8 EBERs

The EBV-encoded RNAs are small nuclear non-polyadenylated RNAs, and are the most abundant EBV RNAs in latently infected cells (Arrand and Rymo, 1982). EBER1, 166

nucleotides in length, is transcribed by RNA polymerase II and III, and EBER2, 172 nucleotides in length, is transcribed by RNA polymerase III (Arrand and Rymo, 1982, Arrand et al., 1989, Rosa et al., 1981, Howe and Shu, 1989, Howe and Shu, 1993). The EBERs localize to the cell nucleus, where they are complexed with the cellular proteins La and EBER-associated protein (EAP) (Fok et al., 2006a, Fok et al., 2006b, Toczyski et al., 1994, Toczyski and Steitz, 1993, Toczyski and Steitz, 1991, Lerner et al., 1981) .

EBER-knockout EBV recombinants are not impaired for primary B cell transformation (Swaminathan et al., 1991) and furthermore, no differences were detected in the growth of the resulting LCLs or in the permissivity of these cells for lytic replication following induction. However, in contrast, it has also been shown that EBERs significantly contribute to the efficient growth transformation of B lymphocytes by enhancing the growth potential of transformed lymphocytes (Yajima et al., 2005). The EBERs have also been shown to confer resistance to interferon gamma (IFNγ) induced apoptosis when expressed in sensitive EBV-negative BL subclones of Akata-BL, Daudi-BL and Mutu-BL (Nanbo et al., 2002), and more recently, it was suggested that EBERs confer resistance to Fas-mediated apoptosis by blocking PKR pathway in human epithelial Intestine 407 cells (Nanbo et al., 2005).

1.10.9 BARTs/CSTs

The BamHI A rightward transcripts (BARTs) or complementary strand transcripts (CSTs) are differentially spliced RNAs detected in BL cells, NPC tumours, latently infected lymphocytes from peripheral blood and primary infected B lymphocytes in culture (Hitt et

al., 1989, Gilligan et al., 1990, Chen et al., 1992, Karran et al., 1992, Brooks et al., 1993, Chen et al., 1999b, Smith et al., 2000). Several putative ORFs have been identified in the *Bam*HI A family of transcripts which may encode proteins in latently infected cells. These include BARF0, encoded in the 3′ ORF in exon 7; an extension of BARF0, RK-BARF0; RPMS1 encoded by an ORF spanning exon 4 and part of exon 5 of some *Bam*HI A transcripts; and A73 encoded by an ORF from components of exons 6 and 7 (Sadler and Raab-Traub, 1995b, Smith et al., 2000, Fries et al., 1997, Kienzle et al., 1999).

Although the function of these transcripts is not fully understood, the finding that they are consistently expressed in all EBV-associated epithelial and B cell tumours and latently infected lymphocytes suggests that they may have important roles in viral persistence. However, recombinant viruses lacking the DNA that encoded the BARTs are capable of efficient transformation of B cells into LCLs, implying the BARTs are dispensable for immortalisation (Robertson et al., 1994, Kempkes et al., 1995b). Most of the viral micro RNAs (miRNAs) expressed in EBV latent infections are derived from the BARTs (Cai et al., 2006, Griffiths-Jones et al., 2006, Grundhoff et al., 2006, Pfeffer et al., 2004). The BARTs miRNAs are thought to be derived mainly from introns prior to splicing of the BART primary transcription (Edwards et al., 2008).

1.10.10 EBV microRNAs

It has been shown that EBV expresses several microRNAs (miRNAs) which may have a role in gene regulation, by either targeting mRNA destruction or by suppressing translation

(Pfeffer et al., 2004). EBV's miRNAs are encoded in two primary transcripts, the BHRF1 transcript which also encodes the BHRF1 orf and the BamHI A rightward transcripts which are a set of long, alternatively spliced transcripts, whose protein coding capacity remains in doubt. The function of the multiple BART miRNA remains to be determined.

1.11 The importance of individual latent genes for transformation: evidence from recombinant EBVs

EBV is very efficient at transforming human resting B cells *in vitro*, leading to the outgrowth of LCLs. Only 11 EBV genes are constitutively expressed in LCLs and much effort has been directed towards identifying the genes that are essential for the virus to transform cell growth. Whereas molecular genetic and biochemical analyses have revealed some relevant biological activities of individual gene products studied in isolation, recombinant EBV genetic analyses allow one to look at individual gene function in the context of the whole viral genome. Such studies have identified which genes are essential for transformation, which are required for optimal transformation and which are dispensable.

1.11.1 Marker rescue of its transformation defective P3HR1 EBV genome

The first genetic analysis was based on marker rescue of the EBV strain present in the P3HR1 cell line (the P3HR1 clone 16 cell line, a hetDNA-negative subclone), itself a subclone of an Africa BL cell line, Jijoye, that originally carried a transformation competent type 2 virus (Cohen et al., 1989, Hammerschmidt and Sugden, 1989). Compared with the prototype strain B95.8, P3HR1 is transformation-incompetent and is deleted for a

DNA segment that contains the entire EBNA2 exon and the last two exons of EBNA-LP, therefore cannot express EBNA2 and can only express truncated EBNA-LP. To generate recombinants, the B95.8 DNA restriction fragment EcoRI A containing EBNA2 and EBNA-LP and substantial flanking DNA was cloned into a shuttle vector. This shuttle vector was introduced into the P3HR1 cell line and the lytic cycle was induced by co-transfection with the viral transactivating gene BZLF1. The virus stock released from these induced cells, which contains both the endogenous P3HR1 virus and recombinants, was able to transform B cells. Experiments using shuttle vectors containing mutation in either EBNA-LP or EBNA2 identified EBNA2 as an essential gene in B-cell transformation. This marker rescue method has since been used to generate more recombinants with sequence insertion or deletion within EBNA2 and identified the essential EBNA2 domains (Cohen and Kieff, 1991, Cohen et al., 1991, Yalamanchili et al., 1996, Yalamanchili et al., 1994) (see detail in EBNA2).

Recombinants that were rescued with wild-type EBNA2 but lacked the BamHI Y1Y2-coded C-terminal domain of EBNA-LP have been generated by marker rescue of P3HR1 (Mannick et al., 1991). Such recombinants were competent for transformation but LCL growth was severely impaired compared with LCLs transformed with recombinants carrying a wild-type EBNA2 and EBNA-LP. These studies indicate that full length EBNA-LP is not essential for initial transformation but nevertheless is important for efficient growth. EBV recombinant molecular genetic analyses of EBNA-LP have focused on the C-terminal region because mutations in the repeating exons are more difficult to construct.

Because of their genetic location adjacent to the P3HR1 deletion, the EBER1 and 2 genes or BHRF1 could be deleted or inactivated by foreign DNA insertion and incorporated into the DNA construct used to repair the EBNA2 and -LP genes. Each of these shuttle vectors was transfected into P3HR1 cells to generate recombinants. EBER-deleted recombinant EBV made in this way was equivalent to wild-type EBV in efficiency of transformation and competent for lytic replication (Swaminathan et al., 1991). Recombinant virus deleted for BHRF1 was likewise transformation competent and replication competent (Marchini et al., 1991). The recombinants using this approach were limited to genes which could be physically linked to the EBNA2-containing DNA segment which is essential for the restoration of the ability of P3HR-1 to transform primary B cells.

A subsequent approach using second-site homologous recombination allows the introduction of mutations into the EBV genes at distant sites, allowing other transformation-associated latent genes to be analysed genetically (Tomkinson and Kieff, 1992b, Tomkinson and Kieff, 1992a). In this approach, P3HR1 cells were co-transfected with EcoRI A DNA fragment, cloned EBV DNA that is mutated in the gene of interest (second-site DNA) and the BZLF1 expression vector. To select for second-site recombinants, primary B cells are infected with the virus mixture and LCLs that emerge are analysed by PCR to identify and recover second-site recombinants from the LCLs where the resident P3HR1 sequences at the second sites have been replaced by vectored sequence. Studies using this method have revealed that EBNA3A, EBNA3C and LMP1 are essential for B-cell transformation whereas EBNA3B, LMP2A and LMP2B are dispensable

(Tomkinson and Kieff, 1992b, Longnecker et al., 1992, Tomkinson et al., 1993, Kaye et al., 1993).

The limitations of this approach were clearly that it was non-quantitative, the read out being either the presence or absence of transformed foci. Also it depended on analysis of LCL following outgrowth and could not say anything about sequences that might have been present in other cells, i.e., sequences required to initiate rather than maintain transformation.

1.11.2 EBV recombinants using B95.8-derived bacterial artificial chromosomes

A much improved B95.8 based strategy was later developed using a bacterial artificial chromosomes (BACs) system. F-factor sequences for propagation in *E. coli.* and individual antibiotic resistance genes for selection in bacteria and in eukaryotic cells were recombined into B95-8 DNA to generate an EBV BAC. This DNA can be specifically mutated and segregated while maintained as an F-plasmid in bacteria, thus enabling in principle high-throughput screening of mutation of every EBV gene. Recombinant BAC DNA can then be transfected into 293 cells (an adenovirus-transformed epithelial cell line) and lytic cycle induced by co-transfection with BZLF1 and BALF4 expressing vectors to generate recombinant virus stocks. BALF4, the gene encoding the gp110 envelope glycoprotein was required in addition to BZLF1 because B95.8 itself contains a deletion within BamHI A fragment which does not remove BALF4, but reduces its level of expression in lytic cycle. LMP1 deleted EBV recombinants were generated using this approach. Surprisingly EBV mutants which lacked either LMP1's COOH terminus, the transmembrane domains or the entire open reading frame were able to generate proliferating B-cell clones whose sustained

growth was dependent on fibroblast feeder cells. These studies show that LMP1 is not required for the initial stages of blast transformation, but under normal culture condition, is required for sustaining proliferation through autocrine/paracrine growth factors (Dirmeier et al., 2003). Likewise using an EBNA-1 deficient EBV mutant virus, transformation was not completely abrogated but its efficiency was reduced by 10,000 fold. Very interestingly, the transformants carried EBV as an integrated genome thereby dispensing with EBNA1's episomal genome maintenance function (Humme et al., 2003). In order to explore the roles of the EBV Bcl-2 homolog proteins, BHRF1 and BALF1, several viral mutants, which carry single or dually inactivated alleles of BHRF1 and BALF1 were generated (Altmann and Hammerschmidt, 2005). Single $BHFR1^-$ or $BALF1^-$ mutants were equally capable of yielding LCLs but only at a high virus dose. Mutants with both BHRF1 and BALF1 inactivated failed to generate LCLs from resting primary B cells but were able to generate clonal LCLs from pre-activated B cells blasts and maintained their proliferation. These studies indicated that either BALF1 or BHRF1 is essential to establish LCLs but are dispensable for their continued proliferation.

1.11.3 EBV recombinants based on an Akata-EBV-derived BAC

Another group developed another system using Akata-derived BACs for generating EBV recombinants (Kanda et al., 2004). The Akata cell line is derived from an EBV-positive sporadic BL case in Japan. It has a Latency I form of infection but with relatively high spontaneous lytic activity that is considerably enhanced after antibody cross-linking of the cell surface IgG. The same line has another rare property in that it also can generate EBV-loss Akata BL clones *in vitro*. Similar to B95.8-derived BAC, Akata-BAC also contains F-

factor sequence and individual antibiotic resistance genes for selection in bacteria (chloramphenicol resistance gene) and in eukaryotic cells (neomycin resistance gene). Instead of using 293 cells, the Akata-EBV-negative cell line is used as a virus-producing cell line and production of progeny viruses can be induced by using anti-IgG antibodies.

Recombinants carrying either EBER1 alone or EBER2 alone were generated using this system and used to evaluate the contributions of EBER1 and EBER2 to EBV-mediated B-cell growth transformation (Yajima et al., 2005). These studies showed that the recombinant EBV expressing EBER2 alone transformed B cells as efficiently as the recombinant expressing both; in contrast, the transforming ability of the recombinant expressing EBER1 alone was significantly impaired, similar to the level of the recombinant lacking both EBER1 and EBER2. These results showed that EBER2 was important for efficient B-cell transformation, while EBER 1 is dispensable.

1.11.4 EBV Recombinants expressing conditionally activated target proteins

To be able to understand the function of a latent protein not only in initiation of transformation but importantly also in maintenance of transformed phenotype, a 4-hydroxy-tamoxifen (4HT)-dependent mutant estrogen receptor hormone binding domain (ERTM) was fused to the target protein to create a conditionally activated target protein. In the medium without 4HT, this HT-fused protein would be docked, inactivated and destabilized in the cytoplasm. To generate the EBNA3AHT recombinants, type 1 EBNA3AHT cosmid DNA was transfected into P3HR1 cells, together with EcoRI A DNA and the BZLF1 expressing vector. The resultant virus was used to infect resting B cells and

LCL clones which contained only type1 EBNA3AHT-expressing recombinant EBV genomes were isolated and used for further analysis. These experiments showed that the growth of EBNA3AHT-expressing LCLs is 4HT dependent, which indicated that the EBNA3A has a critical role in maintaining the proliferation of LCLs (Maruo et al., 2003). Using the same principle, the parental Akata BAC was used to construct a recombinant EBV that expresses a conditionally active EBNA3C (Maruo et al., 2006). In the presence of 4HT, LCLs expressed the E3C-HT protein and grew like wild-type LCLs, however, in the medium without 4HT, E3C-HT protein slowly disappeared and the LCLs gradually ceased growing. Wild-type EBNA3C expression from an oriP plasmid transfected into E3C-HT LCLs protected the LCLs from growth arrest in medium without 4HT, whereas expression of EBNA3A or EBNA3B did not. These results indicate that EBNA3C has an essential role in cell cycle progress and the growth maintenance of LCLs (Maruo et al., 2006).

1.12 Aims of this thesis

From previous work, we know that recombinant virus with a deletion of the key Cp regulatory regions (including the GRE and EBNA2 response element) and the C1 and C2 exons can transform B cells with wild-type efficiency and Wp is instead adopted as the EBNA promoter and appears to compensate entirely for the lack of Cp. However, the importance of Wp has not been fully studied, because of complication of (i) its existence in multiple copies and (ii) the EBNA-LP repeat region is encoded by BamHI W repeats. So in practice, if one reduces BamHI W repeat copy number, one is reducing the number of available Wp for early transcription and one is reducing the size of the EBNA-LP repeat

domains, which itself might affect EBNA-LP's function. The aim of my thesis is to try and answer the following questions regarding the role of Wp in transformation:

(i) To what extent is the number of W copies important in the initiation of B-cell growth transformation and/or the maintenance of the transformed phenotype? How many W copies are needed for efficient transformation *in vitro*?

(ii) Can we distinguish between an effect of reducing Wp copy number itself and knock-on effects from reducing EBNA-LP size?

(iii) Would a BamHI W deleted virus have any activity in B cells , if the postulated function of EBNA-LP to help initiate B cell growth activity were rendered redundant by pre-activation of B cells into cycle with a mitogen?

(iv) Previous studies have shown that an EBV recombinant carrying two Wp copies with mutated BSAP binding sites failed to transform B cells and inferred that EBV ensured the B-cell specificity of its growth-transforming function by exploiting BSAP as lineage-specific activator of the transforming programme. Therefore the final question to be addressed in this thesis is whether the apparent dependence of EBV transforming function upon BSAP binding sites in Wp in a 2W virus is still apparent when one increases the number of W copies.

Chapter 2. Materials and Methods

2.1 Cell culture

2.1.1 Maintenance of Cell lines

The BL cell lines, Raji, Rael and Namalwa and X50-7 and CD+Oku LCLs were maintained in exponential growth at 37°C, 5% CO_2 in 25cm^2 flasks in RPMI 1640 with L-glutamine (Sigma) supplemented with 10% v/v foetal calf serum (FCS) (Cambrex Biosciences), and 10 µg/ml penicillin and streptomycin (Invitrogen). The HEK 293 cell lines were maintained in exponential growth at 37°C, 5% CO_2 in 100mm dishes in the same medium.

To passage the adherent cell lines (293, fibroblasts and L cells), the medium was removed and the cells rinsed in 1 x PBS. Cells were trypsinised with 1 x trypsin (0.5% w/v trypsin, 0.54 mM EDTA (Invitrogen) at 37^{o}C for 2 minutes. Medium was added to terminate trypsinisation, the culture was then split 1 in 5 and topped up with appropriate medium (DMEM with L-glutamine supplemented with 10% v/v foetal calf serum (FCS) (Cambrex Biosciences), and 10 µg/ml penicillin and streptomycin (Invitrogen))

2.1.2 Cryopreservation of cells

Approximately 10^7 cells were pelleted by centrifugation at 1400 rpm for 5 minutes and resuspended in 1ml freezing mix (50% v/v B cell medium, 40% v/v FCS and 10% v/v dimethyl sulphoxide (DMSO)). Cells were transferred to cryopreservation tubes and frozen overnight at -80ºC in a freezing box surrounded by sponge soaked in isopropanol. Cells were then moved to the vapour phase of a -180ºC liquid nitrogen freezer for long-term storage.

2.1.3 Recovery of cells from liquid nitrogen

Cells were thawed quickly in a 37ºC water bath and transferred to a 15ml tube. 10ml pre-warmed B cell medium was added gradually to the cells dropwise to dilute DMSO. Cells were pelleted by centrifugation at 1400rpm for 5 minutes, resuspended in the appropriate medium and transferred to 25cm^3 flasks and incubated at 37 ºC, 5% CO_2.

2.1.4 Preparation of CD19 positive B cells from whole blood

Blood samples were diluted with an equal volume of 1 x PBS, mixed well and layered onto lymphocyte separation medium (Lymphoprep, Axis Shield) slowly and carefully. This was then centrifuged at 1800rpm for 30 minutes and slowed without the brake. Unfractionated mononuclear (UM) cells, which formed a distinct band at the interface between serum and separation medium, were collected and washed three times with 1 x PBS using centrifugation at 1600rpm for 10 minutes, 1200rpm for 10 minutes and 1000rpm for 10 minutes. UM cells were resuspended in RPMI/1% v/v FCS, counted and the number of B cells estimated based on the assumption that 5% of UM cells were B cells. CD19 Dynabeads (Invitrogen), magnetic polymer beads coated in anti-CD19 monoclonal antibody, were used to positively select B cells. The required number of CD19 Dynabeads (approximately 4 beads per B cell) were added to UM cells at a concentration of approximately $1x10^7$ beads per ml and incubated at 4 ºC for 30 minutes with a rocking and rolling motion. Beads were exposed to a magnet for 2 minutes and CD19-negative cells (non-B cells) were removed from the bead-bound B cells. Beads and bound B cells were washed five times in 10ml RPMI/1% v/v FCS with exposure to a magnet for 2 minutes each time. To detach cells from the beads, Detachabead CD19 (Invitrogen) was added at a

concentration of 10µl per 10^7 B cells in 100µl of RPMI/1% v/v FCS and incubated at room temperature for 45 minutes. After incubation, the cells were separated from the beads with 3 washes of 8ml RPMI/1% v/v FCS with exposure to a magnet for 2 minutes between washes

2.2 Bacteriology

2.2.1 Bacteria growth media

Lennox Broth (LB): a solution of 2% w/v LB Broth base (Fisher) in deionised water, sterilised by autoclaving at 121^{o}C, 15psi for 15 minutes.

LB-agar: a solution of 1.5% w/v agar (Fisher) in LB, sterilised by autoclaving at 121^{o}C, 15psi for 15 minutes.

Preparation of LB-agar plates

The LB-agar was melted in a microwave, cooled to 50^{o}C with the addition of the appropriate antibiotics (see Table 2.1) and poured into petri dishes and left to solidify. Plates were stored at 4^{o}C for up to 1 week. Before use the plates were dried in a 37^{o}C incubator for 30 minutes.

Table 2.1 Working concentrations of antibiotics

Antibiotics	**Working concentration**
Ampicillin	100µg/ml
Chloramphenicol	15 µg/ml
Tetracyclin	10 µg/ml
Kanamycin	50 µg/ml

2.2.2 Blue/white colony selection

Blue/white colony screening is a method which can be used for detection of successful ligation in vectors containing the lacZ (B-galactosidase) gene. 80µl of 2% w/v 5-bromo-4-chloro-3-indoyl-β-D-galactoside (X-gal) and 20µl of 100mM isopropyl thio-β-D-galactoside (IPTG) were spread onto L-agar/ampicillin plates and left for 30 minutes to infuse into the agar before the transformed bacteria were plated out. Transformants containing vector only appear as blue colonies while transformants with recombinant vectors appear as white.

2.2.3 Bacterial strains

DH5α (*supE44* Δ*lacU169 (*Φ*80 lacZ* Δ*M15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*) – used for standard DNA cloning

DH10B (Δ (*mrr-hsd RMS-mcrBC*) *mcrA recA1*) – used for maintaining BACs

BJ5183 (*recB recC sbcB sbcC endA galK met thi-1 bioT hsdR rpsL(strR))* – used for homologous recombination of BACs

2.2.4 Preparation of competent *E. coli*

Simple and efficient method (SEM) competent *E. coli*

A single colony of the appropriate E. coli strain was picked from an agar plate and cultured overnight in 10ml LB in a 37^{o}C incubator with vigorous shaking. 100µl overnight culture was then used to inoculate 250ml LB supplemented with 5mM $MgCl_2$ and 5mM $MgSO_4$, which was then incubated at 18ºC with gentle shaking until the absorbance at 600nm (A_{600}) reached 0.6 (approximately 30 hours of growth). The culture was incubated on ice for 10

minutes and transferred to 50ml tubes before being centrifuged at 3000rpm for 10 minutes at 4 ºC. The cell pellet was resuspended in 80ml ice cold filtered TB buffer (10mM piperazine-1,4-bis[2-ethanesulphonic acid] (PIPES), 15mM $CaCl_2$, 250mM KCl, 55mM manganese chloride), incubated on ice for 10 minutes before being centrifuged at 3000rpm for 10 minutes at 4 ºC, and then resuspended in 20ml ice cold TB-DMSO (7%v/v). The competent cells were dispensed into 40µl aliquots in 1.5ml Eppendorfs, snap frozen in liquid nitrogen and stored at –80^{o}C.

Preparation of TFB competent cells

Bacterial cells were seeded into 4ml of LB and cultured overnight in a 37^{o}C incubator with vigorous shaking. The overnight culture (100 µl) was then used to inoculate 30ml LB which was then incubated at 37ºC with gentle shaking until the absorbance at 600nm (A_{600}) reached 0.6. The culture was then transferred to a pre-cooled sterile 50ml tube and incubated on ice for 30 minutes followed by centrifugation at 3000rpm for 10 minutes at 4 ºC. The bacterial pellet was then resuspended in 10ml of ice cold TFB buffer (10mM K-MES pH6.2 [2-(N-morpholino)ethanesulfonic acid (MES) buffer. Adjust the pH to 6.2 using concentrated KOH], 50mM $CaCl_2$, 45mM $MnCl_2$, 100mM $RbCl_2$) and incubated for 30 minutes on ice, followed by centrifugation again as above. The competent cells were then resuspended in 2.4ml of ice cold TFB buffer and immediately used as described in section 2.2.4 (a).

Preparation of electroporation competent *E.coli*

A single colony of DH10B was picked from an agar plate and cultured overnight in 4ml LB in a 37^{o}C incubator with vigorous shaking. The overnight culture (200µl) was then used to inoculate 200ml LB and grown at 37ºC with shaking until the absorbance at 600nm (A_{600})

reached 0.7. The culture was incubated on ice for 10 minutes and transferred to four 50ml tubes before being centrifuged at 3000rpm for 10 minutes at 4 ºC. The cell pellets were washed twice using 50ml ice cold 10% v/v glycerol and centrifugation at 3000rpm for 10 minutes at 4 ºC. The cells were then pooled and washed again with 30ml 10% v/v glycerol and then resuspended in 400µl ice cold 10% v/v glycerol. The competent cells were dispensed into 20µl aliquots in 1.5ml Eppendorfs, snap frozen in liquid nitrogen and stored at –80^{o}C.

2.2.5 Bacterial transformation

Heat shock

The SEM competent cells were thawed on ice and 100-200µl of competent cells were removed to a pre-chilled 1.5ml Eppendorf. DNA was added to the competent cells and incubated on ice for 20 minutes before being heat shocked at 42 ºC for 2 minutes followed by 2 minutes incubation on ice. To allow the cells to recover, 1ml of LB was added and the cells were incubated at 37 ºC for 1 hour. The cells were then centrifuged and the supernatant tipped off to leave approximately 100µl in which the cells were resuspended. The entire volume was then plated out on a LB-agar plate with antibiotics and grown overnight at 37 ºC.

Electroporation

The electroporation competent cells were thawed on ice before DNA was added. The sample was then removed to a pre-cooled 1mm gap cuvette (Geneflow) and incubated on ice for 10 minutes. The sample was then electroporated using 25µFD and 1.8kV. The eletroporated cells were then removed to a 1.5ml eppendorf with 1ml LB and incubated at

37 ºC for an hour to recover. The cells were then centrifuged and the supernatant tipped off to leave approximately 100µl in which the cells were resuspended. The entire volume was then plated out on a L-agar plate with antibiotics and grown overnight at 37 ºC.

2.3 Preparation of DNA

2.3.1 Small scale purification of plasmid DNA

Single colonies were picked from an agar plate using sterile yellow tips and incubated in 3ml of LB supplemented with the appropriate antibiotic. Cultures were grown overnight at 37^{o}C with shaking. Next day, 1.5ml of culture was transferred to a 1.5ml Eppendorf and the cells pelleted by centrifugation at 13000rpm for 30 seconds. The plasmid DNA was extracted using a GFX-Microplasmid Prep. kit (Amersham Biosciences) according to the manufacturer's instructions. The DNA was eluted in a final volume of 50µl elution buffer and 5µl of DNA was digested with selected restriction enzymes and subjected to agarose gel electrophoresis to confirm that the plasmid was correct.

2.3.2 Alkaline lysis

For the preparation of DNA from bacteria colonies transformed with recombinant EBV BACs, an alternative alkaline lysis method was employed. Transformed colonies were picked and spread as a lawn onto ¼ of an agar plate per colony. These were grown overnight at 37°C and the following day, the lawn was scraped off using sterile wooden tooth picks and gently resuspended into a large microtube containing 200µl of ice cold resuspension buffer (25mM Tris-HCl pH 8.0, 10mM EDTA, 50mM glucose) containing 0.2µl RNaseA. 300µl of freshly made lysis buffer (0.2M sodium hydroxide, 1% SDS) was

then added and the microtube gently inverted five times to ensure complete mixing. Lysis was then allowed to occur for five minutes at room temperature. Following lysis, precipitation of chromosomal DNA, protein and cellular debris occurred by the addition of a neutralisation buffer (5M potassium acetate, glacial acetic acid at 115ml per litre) with tubes inverted gently several times and then left for 10 minutes on ice. Sample tubes were then centrifuged for 20 minutes at 13000rpm and the supernatant transferred to a new tube, followed by a second identical centrifugation step. The supernatant was then transferred to fresh microtubes and DNA precipitated by the addition of 0.6 volumes of isopropanol and incubation on ice for 10 minutes. Precipitated DNA was pelleted by centrifugation for 20 minutes at 13000rpm, the supernatant aspirated and DNA washed with 70% ethanol. After a further 5 minute spin and aspiration of the ethanol, pellets were air dried and resuspended in the desired volume of 1X TE buffer.

2.3.3 Large scale purification of plasmid DNA

A single *E.coli* transformant was picked from an agar plate and incubated in 5ml LB, containing the appropriate antibiotic overnight at 37^{o}C with shaking. Next day 1ml of the starter culture was used to inoculate 25ml L-broth (midiprep)/200ml L-broth (maxiprep), supplemented with the appropriate antibiotics, and grown overnight at 37^{o}C with shaking. Plasmid DNA was purified using the Jetstar Midiprep/MaxiPrep kit (Genomed) according to manufacturer's instructions. DNA was resuspended in 100µl (midiprep)/400µl (maxiprep) TE buffer and the concentration determined by spectrophotometry.

2.4 Cloning techniques

2.4.1 Restriction enzyme digestion of DNA

DNA was digested with specific restriction enzyme in the appropriate buffers (Roche or New England Biolabs) at the required temperature. For the digestion of the relatively small vectors or inserts, a 20µl reaction consisting of 1µg DNA, 2 units of specific restriction enzyme, 1X appropriate buffer was set up and incubated at the optimal temperature for 2 hours. Double or triple digests were usually carried out in a single reaction in a buffer that gave optimal activity for each enzyme with appropriate enzymes that were sufficiently optimal in a single reaction buffer.

2.4.2 Agarose gel electrophoresis

The percentage agarose gel used depended upon the size of the expected DNA product but was generally between 0.8% and 2% w/v (Molecular Biology Grade, Eurogentec) in 1X TBE buffer (90mM Tris-borate, 2mM EDTA, pH8.0). The DNA solution was mixed with 6 x gel loading buffer (0.25% w/v xylene cyanol, 0.25% w/v bromophenol blue, 30% v/v glycerol) loaded onto the gel alongside a 1kb DNA ladder (Invitrogen) and the DNA fragments separated by electrophoresis at 90-120 volts. After electrophoresis the gel was stained in a bath of 1 x TBE containing 0.5µg/ml ethidium bromide for 3 minutes and the DNA visualised using a UV transilluminator and the fragment sizes determined by comparison with the 1kb ladder.

2.4.3 Field inversion gel electrophoresis (FIGE)

BAC DNA digested with EcoRI and HindIII was mixed with 6 x gel loading buffer loaded onto a 0.8% agarose gel, alongside a 2kb DNA ladder (Invitrogen). Field inversion gels were run for 20 hours in a tank with re-circulating buffer with a FIGE Mapper (Biorad) power supply using the following programme: 0.8 sec at 135 V in the forward direction, 0.8 sec at 90 volts in the reverse direction. After electrophoresis the gel was stained in a bath of 1 x TBE containing 0.5µg/ml ethidium bromide for 3 minutes and the DNA visualised using a UV transilluminator and the fragment sizes determined by comparison with the 2kb ladder.

2.4.4 Gel extraction of DNA fragments

PCR products or restriction enzyme digests were separated by agarose gel electrophoresis. The ethidium bromide stained gel was examined on a UV transilluminator and the required product band was excised and the DNA extracted using Qiagen Gel Extraction kit according to the manufacturer's instructions. The DNA was eluted from the column in 50µl sterile deionised water and stored at –20^{o}C until required.

2.4.5 Conventional DNA PCR

PCR amplification was used to amplify the desired gene from the plasmids which contained the relevant cDNA. A 50µl reaction mix was set up in a 0.5ml thin-walled microfuge tube containing 20-40ng DNA, 3.5 units of high fidelity Taq polymerase (Roche), 200µM each of dATP, dCTP, dGTP, dTTP , 1µM of the gene-specific (forward and reverse) primers (Alta Bioscience) (see Table 2.2), 1.5mM $MgCl_2$ (ABgene) and 1x polymerase reaction

buffer (75mM Tris-HCl pH8.8, 20mM ammonium sulphate ($(NH_4)_2SO_4$), 0.01% v/v Tween20) (ABgene). An Eppendorf Thermocycler was used with a hot start at 94^{o}C for 5 minutes followed by 30–35 cycles of 30 sec. at 95^{o}C to denature the DNA strands, 30sec. to 1 minute at 45^{o}C-60^{o}C to allow the primers to anneal to the DNA and 2-4 minutes at 72^{o}C to allow extension of the product. The annealing temperature and incubation time at each stage varied according to the specific primers and product size of each PCR (see Table 2.2). The PCR products were separated by agarose gel electrophoresis.

2.4.6 Ligation

1µl of insert DNA from gel extraction and 1 µl of restriction enzyme digested vector DNA were first run on an agarose gel to estimate the concentration. A 20µl ligation reaction was set up consisting of a 3:1 molar ratio of insert DNA to vector DNA, 1x rapid ligation buffer and 1µl T4 rapid ligase (Roche) and was incubated at room temperature for 15 minutes and then used to transform SEM competent E. coli cells.

2.4.7 Sequencing

DNA was sequenced using a Big Dye Version 3 sequencing kit (Applied Biosystems). Approximately 200ng of plasmid DNA was put into a 20µl volume sequencing reaction with 3.2pmol of sequencing primer. 4µl Terminator Ready Reaction Mix (Applied Biosystems) and 1 x sequencing buffer (40mM Tris-HCl pH9, 1mM $MgCl_2$). An Eppendorf Thermocycler was used to denature the samples at 95^{o}C for 2 minutes followed by 25 cycles of 95^{o}C for 10 seconds, 50^{o}C for 10 seconds and 60^{o}C for 4 minutes. After PCR amplification, the DNA was precipitated by the addition of 64µl 96% ethanol and 2µl

0.25M EDTA and incubation at room temperature for 15 minutes. The DNA was pelleted by centrifugation at 13000rpm for 20 minutes, washed with 70% v/v ethanol, air-dried and resuspended in 10µl HiDi formamide (Applied Biosystems). The samples were subjected to automated sequence analysis using a 3700 DNA Analyser (Applied Biosystems) provided by the Functional Genomics Laboratory, School of Biosciences, University of Birmingham.

2.5 Generation of recombinant viruses using the EBV BAC system

2.5.1 Recombination

Recombination in *E. coli* BJ5183

Recombinant virus genomes were generated by using the EBV bacterial artificial chromosome (BAC) cloning system (Delecluse et al., 1998). Shuttle vectors with homologous regions and modified regions were first made (details are described in chapter 3) and allelic exchange was performed in E. coli strain BJ5183.

BACs which either contained 2 copies of BamHI W fragments (2W BAC) or were deleted for BamHI W fragments (0W BAC) used as the parental BACs (Tierney et al. 2007. 10µg of shuttle vector DNA were first linearised overnight with 20 units of the appropriate restriction enzyme in a 100 µl reaction. 10µl of the reaction and 7µl of DMSO were then added to 200µl of TFB competent BJ5183 containing the parental BAC which were then transformed using the heat shock method (see section 2.2.5 (a)), plated out on a tetracycline/chloramphenicol agar plate and grown overnight at 37°C. For screening, colonies were picked and spread as a lawn onto ¼ of an agar plate containing appropriate antibiotics and grown overnight at 37ºC. DNA was extracted by alkaline lysis mini-prep and digested with appropriate restriction enzymes. The whole digest was loaded on to a

0.8% w/v agarose gel (Fisher) with 1kb and 2kb ladder (Roche) loaded on both sides, and subjected to electrophoresis at 55V for 36 hours. After eletrophoresis, the gel was stained with ethidium bromide and the DNA visualised using a UV transilluminator. The fragments were compared to control digests with the parental BACs and the fragment sizes predicted from maps made with MacVector software (Accelrys).

Recombination was used to remove the tetracycline cassette which was flanked by Flp recombination target sites. 50ng of plasmid pcp20 (containing Flp recombinase gene and a temperature-sensitive replication origin) and 7µl of DMSO were added to 200µl of TFB competent cells containing the tet^+ recombinant. The cells were then transformed using the heat shock method, recovered at 30ºC and plated out on a chloramphenicol/ampicillin agar plate and grown for 1-2 days at 30ºC to allow recombination to occur. Colonies were then picked and grown at 37 ºC for 8 hours in 4ml of LB containing chloramphenicol only, followed by plating out onto a chloramphenicol agar plate and grown at 42 ºC overnight in order to inactivate and discard the pcp20 plasmid. After Flp recombination, colonies were screened by restriction enzyme digestion. One correct colony was chosen and DNA was extracted by alkaline lysis mini-prep and transformed into the DH10B rec A- stable cell line, by electroporation (see section 2.2.5 (b)).

Recombination using pDK46 plasmid

pDK is a plasmid carrying the red recombinase under the control of an arabinose-inducible promoter, a temperature-sensitive replication origin and ampicillin resistance gene. pDK46 plasmid was transformed into chemically competent DH10B containing the parental BACs (2W or 0W) by heat shock and cells were plated out on a chloramphenicol/ampicillin plate

and grown overnight at 30 ºC. A single colony was picked from the plate and cultured overnight in 4ml LB at 30 ºC. 200 µl of the culture were then used to inoculate 200ml LB supplemented with 20mM L-arabinose. This culture was then grown at 30ºC with gentle shaking until the absorbance at 600nm (A_{600}) reached 0.6 and electrocompetent cells were made as described in section 2.2.4 (c). 3 µg of linearised shuttle vector was then transformed in to the electrocompetent DH10B-BAC-pDK46 cells by electroporation (see section 2.2.5 (b)) and cells then recovered in arabinose containing media for 3 hours at 30ºC, during which time recombination occurred. The cells were then plated out on a chloramphenicol/tetracycline plate and grown overnight at 42ºC and pDK46 plasmid was lost at this temperature. Colonies were screened as described above. If required the tetracycline cassette was removed using flp recombination as described in the previous section.

2.5.2 Large scale purification of BAC DNA

500ml of LB containing the appropriate antibiotics were seeded with *E.coli* transformant either directly from an agar plate for from a glycerol stock of the bacteria and grown overnight at 37ºC with shaking. A nucleobond AX BAC 100 kit (Macherey-Nagel) was used to purify recombinant EBV BAC DNA according to the manufacturer's instructions. DNA was resuspended in 50µl 1X TE buffer.

2.5.3 Transfection of BAC into 293 cells and clone selection

One day before transfection, $8x10^5$ 293 cells were plated in a 6-well plate so that the cells were 60-80% confluent at the time of transfection. BAC DNA (5µg) and 10µl of

Lipofectamine 2000 (Invitrogen) were diluted in 250µl of Opti-MEM (Invitrogen) separately and incubated at room temperature for 5 minutes, mixed together and incubated for further 20 minutes. The complexes were then added to the 293 cells and incubated for 3 hours before 3ml of RPMI1640 10% v/v FCS were added to the cells. The following day, the transfected cells were trypsined from the 6 well-plate and transferred to a 150mm ^{2} dish in 30ml medium containing 100µg/ml hygromycin. Visible colonies were removed from the dish to 96 well plates after 3-4 weeks and the cultures gradually expanded from 96 well plates to 100mm dishes while maintaining drug selection.

2.5.4 Preparation and purification of virus stocks

293 cell clones carrying recombinant EBV genomes were plated into 6-well plates 2 days before transfection so that the cells were 60-80% confluent at the time of transfection. 0.5 µg of BZLF1 expressing plasmid (p509), 0.5µg BALF4 plasmid (pRA) and 6 µl of Lipofectamine (Invitrogen) were diluted in 100 µl of Opti-MEM (Invitrogen) and incubated at room temperature for 30 minutes before being further diluted with 900µl of Opti-MEM. The complexes were then added to the 293 cells and incubated for 3 hours before 1ml of RPMI 1640 10% v/v FCS was added. The cultures were then incubated for 72 hours.Virus containing culture supernatant was then harvested, centrifuged at 1400rpm for 4 minutes and filtered using a 0.45µm filter to remove cellular contaminants.

To concentrate and purify virus stocks, 10.5ml of supernatant was added to a 15ml Beckman Polyallomer untracentrifuge tube and underlaid with 1ml Optiprep (Axis Shield) diluted 1:1 with dilution buffer (0.85% (w/v) NaCl, 60mM Hepes-NaOH, pH 7.4). Tubes

were weighed to balance and centrifuged in a SW40 rotor at 20,000rpm for 2 hours with low brake. Most of the supernatant was removed by aspiration and discarded and the remaining contents, consisting of 1ml of supernatant and concentrated virus particles on a cushion of Optiprep, were then mixed and transferred to 4.9ml Beckman tube and topped up with Optiprep diluted 1:3 with dilution buffer and centrifuged in a VTi65 vertical rotor at 64,000rpm for 2 hours and 40 minutes with low brake. A self-generated gradient of Optiprep formed in centrifugation and a visible virus band was harvested with a syringe and needle by tube puncture. The virus stock was stored in aliquots at -80°C.

2.5.5 Quantification of virus titres

To quantify the virus stock, an equal volume of lysis buffer (100µg/ml proteinase K (Roche), 10mM Tris-HCl pH8.8, 1.5mM $MgCl_2$, 50mM KCl, 0.1% (v/v) Triton X-100) was added to the viral supernatant and incubated at 55ºC for 1 hour followed by 99 ºC for 10 minutes to heat inactivate the proteinase K. Q-PCR was then carried out for the EBV DNA polymerase gene (BALF5). DNA standards were made from Namalwa, a cell line known to contain 2 integrated EBV genomes per cell. Namalwa DNA was diluted to 132ng/µl which corresponds to 40,000EBV genomes/µl, assuming one Namalwa cell contains 6.6pg of DNA and 2 EBV genomes. Serial dilutions were made to give standards with 10,000, 4000, 400, 100, 20 and 1 EBV genomes/µl. A 25µl reaction volume was set up in the wells of a 96 well Optical Reaction Plate (Applied Biosystems) consisting of 5µl virus lysate (or DNA standards), 1x Taqman Universal PCR Master Mix (Applied Biosystems), 200nM of each EBV Pol primer, 200nM of EBV pol probe (see Table 2.3). Thermocycling and fluorescence detection were carried out in an ABI Prism 7500

Sequence Detection System (Applied Biosystems). The samples were heated to 50^{o}C for 2 minutes, 95^{o}C for 10 minutes to activate the Amplitaq Gold, followed by 40 cycles of denaturation at 95^{o}C for 15 seconds and primer annealing and extension at 60^{o}C for 1 minute. Data were analysed using Applied Biosystems Sequence Detection Software version 1.4.

2.6 Characterization of Infection

2.6.1 Binding assays

1x10^{6} primary B cells were exposed to purified virus stocks at an moi 50 for 3 hours at 4ºC, then the cells were washed 3 times with cold PBS to remove unbound virus. DNA was extracted from the cell pellet using a DNAeasy Tissue kit (Qiagen) according to the manufacture's instructions. The DNA was eluted from the columns in 100µl 1X TE buffer. A multiplex Q-PCR allowed amplification of the EBV polymerase (Pol) gene with a FAM-labelled probe and the cellular gene, β2m with a VIC-labelled probe simultaneously. Primers and probes were designed using Primer Express software (Applied Biosystems) and are detailed in table 2.3. Namalwa BL DNA, known to contain 2 integrated EBV genomes per cell, was included in the PCR as standards. PCR reactions contained 50-100ng DNA, 1x Taqman Universal mastermix (Applied Biosystems), 200mM of EBV Pol primers, 200nM EBV Pol probes (Eurogentec), 60nM of β2m primers and 100nM β2m probes. The samples were heated to 50 ºC for 2 minutes, 95 ºC for 10 minutes to activate AmpliTaq Gold, followed by 40 cycles of 95 ºC for 15 seconds and 60 ºC for 1 minute. Fluorescent signals were detected by an ABI Prism 7500 Sequence Detection System (Applied

Biosystems) and data were analysed using Applied Biosystems Sequence Detection Software version 1.4.

2.6.2 Internalisation assays

To assay virus internalisation, $4x10^6$ primary B cells were exposed to purified virus stocks at an moi 50 overnight at 37 ºC, then treated with chymotrypsin (2mg/ml) at 4 ºC for 1 hour to strip off the viruses bound to the cell surface. 1ml PBS containing 1.25mM PMSF and 5% w/v BSA was added to the cells to stop further proteolysis. Cells were washed twice with PBS and DNA was extracted from the cell pellet as described above and subjected to Q-PCR assay as described above to determine the number of internalised genomes per cell.

2.6.3 Quantification of EBV transcripts

RNA isolation

To assay viral gene transcription, $10x10^6$ primary B cells were exposed to purified virus stocks at an moi 50 overnight at 37 ºC and $3x10^6$ cells were harvested at 1, 2 and 3 days post-infection. Total RNA was isolated from infected cells using a Macherey-Nagel Nucleospin II kit (Fisher) according to the manufacturer's instructions. RNA was eluted in a final volume of 50µl and the concentration determined using a Nanodrop ND-1000 spectrophotometer (Labtech International). RNA was then diluted to the concentration of 100ng/ µl and stored at -80ºC.

Reverse transcription

RNA was reverse transcribed to cDNA to study RNA expression. 200ng RNA were denatured by heating at 90ºC for 2 minutes and then plunged into ice. Denatured RNA was

reverse-transcribed in a 20µl reaction containing 5 units of avian myeloblastosis virus reverse transcriptase (AMV-RT) (Roche), 200µM mix of dNTPs, 1µM of the mix of 3' EBV cDNA primers (Alta Bioscience) (see Table 2.4) and 1x reaction buffer (50mM Tris-HCl pH8.5, 30mM KCl, 8mM $MgCl_2$, 1mM dithiothreitol (DTT) (Roche). This mixture was incubated at 42ºC for 90 minutes, followed by 5 minutes at 90ºC to heat inactivate AMV-RT. cDNA was diluted to a final volume of 40µl and stored at -20ºC.

Q-RT-PCR to detect EBV transcripts

Q-RT-PCR was used to detect EBV transcripts. Primers and probe combinations for specific transcripts are listed in Table 2.4. All EBV-specific probes were FAM labelled, while the GAPDH probe was VIC-labelled, therefore the quantity of the test gene transcripts can then be normalised to a cellular control gene (GAPDH). 25ng of cDNA from above was tested per well of a 96-well plate in a 25µl reaction containing 1x Taqman Universal PCR mastermix (Applied Biosystems), primers and probe (Table 2.4) and 1x GAPDH mix of primers and probe (Applied Biosystems). All samples were analysed in duplicate. The samples were heated to 50^{o}C for 2 minutes, 95^{o}C for 10 minutes to activate the Amplitaq Gold, followed by 40 cycles of denaturation at 95^{o}C for 15 seconds and primer annealing and extension at 60^{o}C for 1 minute. Dilutions of cDNA from appropriate reference cell lines were included for each PCR in order to generate standard curves (Table 2.4).

2.6.4 Immunofluorescence cell staining

$1x10^6$ primary B cells were exposed to purified virus stocks at an moi 50 overnight at 37 ºC and harvested at 2 days post-infection. Cells were washed twice with PBS and resuspended

in 200µl PBS. 5µl of the cells were then spotted onto a 10-well microscope slide (Hendley-Essex). The slide was left at room temperature for the liquid to dry out before being fixed in fixing solution (1:1 methanol/ acetone) at -20^{o}C for 30 minutes. 30µl of 10%v/v heat inactivated goat serum (HINGS) were added to each well on the slide and incubated at room temperature for 20 minutes for blocking. The blocking solution was then drained off and replaced with 30µl of primary antibody (Table 2.5) and the slide was incubated for 30 minutes at 37^{o}C in a moist plastic box. The slide was then washed twice for 5 minutes in PBS in staining jar with magnetic flea. 30 µl of Alexa Fluor 488 conjugated secondary antibody (Invitrogen) diluted at 1 in 1000 in10%v/v HINGS were added to each well immediately after washing and the slide incubated at 37 ^{o}C for 1 hour in a moist plastic box. The slide was then washed twice again in PBS and a drop of DABCO solution containing DAPI (0.5µg/ml) was added to each well followed by a cover slip gently placed on the slide.

Table 2.5 Primary antibodies used to detect EBV latent proteins by immunofluorescence cell staining

Target protein	Antibody	Origin	Dilution
EBNA2	PE2	Mouse	1 in 200
EBNA-LP	JF186	Mouse	1 in 10

2.6.5 Western Blotting

Preparation of protein samples

Approximately 10^{7}cells were harvested, washed twice with PBS and lysed in 200µl urea buffer (9M urea, 50mM Tris-HCl pH 7.5). Samples were then sonicated for 20 seconds to

disrupt genomic DNA. The concentration of the protein was determined using a Biorad kit according to the manufacturer's instructions. The protein samples were diluted in urea gel sample buffer (62.5 mM Tris pH6.8, 4% w/v SDS, 5% β-mercaptoethanol, 0.01% w/v bromophenol blue, 5M urea, 10% glycerol) to an appropriate concentration and denatured at 100^{o}C for 2 minutes prior to sodium dodecyl sulphate– polyacrylamide gel electrophoresis (SDS-PAGE) or frozen at -20 ^{o}C.

SDS-PAGE

Proteins were separated by SDS-PAGE gels using a Bio-Rad Mini Gel Tank. SDS-PAGE gels were made in two phases, according to the method of Laemmli (Laemmli, 1970). Resolving gels were made with10% acrylamide (2.5ml H_2O, 7.5ml Protogel acrylamide mixture, 4.2ml 1.5M Tris-HCl pH8.0, 0.15ml 10% w/v SDS, 0.025ml 10% w/v APS and 0.015ml TEMED) and poured immediately. 300µl water-saturated butanol were applied immediately on top to give a uniform surface. The gel was allowed to set for 45 minutes before the butanol was poured off and the gel surface washed three times with distilled water. A stacking gel consisting of 4% acrylamide (5.8ml H_2O, 1.5ml Protogel acrylamide mixture, 2.5ml 0.5M Tris-HCl pH6.8, 0.1ml 10% w/v SDS, 0.05ml 10% w/v APS and 0.03ml TEMED) was made and pipetted on top of the resolving gel. Gel-forming combs were inserted into the stacking gel and the gel left to polymerise for 30 minutes. The gel was placed into the Bio-Rad running tanks and submerged in running buffer (25mM Tris-HCl pH8.3, 192mM glycine, 0.1% w/v SDS). 20µg of protein samples were loaded into the wells alongside 6µl SeeBlue® Plus2 pre-stained marker (Invitrogen). The proteins were run into the stacking gel at 60V until the bands had passed the stacking gel and run into the

resolving gel, after which the voltage was increased to 120V until the samples reached the base of the gel.

Western blotting

After SDS-PAGE, protein samples were blotted onto a nitrocellulose membrane (BioTrace^{TM} NT 0.2µm nitrocellulose filters, PALL Life Sciences). A blotting cassette was set up in which the gel and the nitrocellulose membrane were sandwiched between pieces of 3MM Whatman filter paper and sponges pre-soaked in blotting buffer (25mM Tris-HCl pH8.3, 192mM glycine, 20% v/v methanol). The blotting cassette was placed in Bio-Rad Trans-blot^{TM} mini-cell blotting chambers, submerged in blotting buffer and protein transfer carried out at 11V for 18 hours in a cold room with an ice cooling unit present in the blotting chamber.

After 18 hours blotting, the membrane was then removed gently from the cassette and washed briefly in distilled water before being stained with Ponceau S stain (1% w/v Ponceau S (Sigma) in 3% trichloroacetic acid) for 3-5 minutes to visualise the marker and the sample proteins. The membrane was washed twice in PBS with 0.1% v/v Tween80 until the stain had been removed and then incubated in blocking solution (5%w/v skimmed milk powder in PBS-0.1% Tween) for 1 hour at room temperature with shaking. The membranes were finally sealed in plastic bags with 10ml of primary antibody (Table 2.6) diluted in blocking solution and incubated with shaking overnight at 4^{o}C.

Following incubation, the primary antibody solution was drained off (the antibody was stored at –20^{o}C and reused several times) and the membrane washed twice for 10 minutes in 1 x PBS-0.1% Tween20. The membrane was then sealed in a bag with the secondary peroxidase-conjugated antibody, diluted at 1 in 1000 in blocking solution, and incubated with shaking at room temperature for 2 hour. The secondary antibody was then discarded and the membrane washed six times for 15 minutes each in 1 x PBS-0.1% Tween20.

Table 2.6 Primary antibodies used to detect EBV latent proteins by Western Blotting

Target protein	Antibody	Origin	Dilution	Reference
EBNA1	1H4	Rat	1 in 50	(Grasser *et al*. 1994)
EBNA2	PE2	Mouse	1 in 50	(Young *et al*. 1989)
EBNA-LP	JF186	Mouse	1 in 50	(Finke *et al*. 1987)
EBNA3A	HG/RS22	Mouse	1 in 50	(Maunders *et al*. 1994)
EBNA3C	E3CA10	Mouse	1 in 50	(Maunders *et al*. 1994)
LMP1	CSI-4	Mouse	1 in 25	(Rowe *et al*. 1987a)
LMP2A	14B7	Rat	1 in 50	(Fruehing *et al*. 1996)
BHRF1	5B11	Mouse	1 in 1000	Millipore

To visualise the proteins, the membrane was incubated in equal amounts of ECL reagents A and B (Amersham) for 1 minute before being wrapped in Saranwrap and exposed to Kodak X-Omat AR film in an autoradiograph cassette. Exposure times varied from 10 seconds to 30 minutes depending upon the strength of the signal. The membrane was washed twice

with PBS-0.1% Tween and either wrapped wet in Saranwrap and stored at 4 ^{o}C or was re-probed with an alternative primary antibody after additional blocking.

Chapter 3. The role of EBV BamHI W copy number in B cell growth transformation

The BamHI W promoter, Wp, is present in each BamHI W repeat in the EBV genome and is selectively activated immediately after infection. The number of BamHI W repeats, and therefore the number of Wp copies (from 3-12 copies) varies between EBV strains. It has been suggested that Wp may be designed to function particularly well in the quiescent environment of a resting B cell due to its presence in multiple copies. In previous studies, recombinant viruses carrying only two copies of Wp were able to transform B cells *in vitro*, but the transformation efficiency was much lower compared to the parental virus carrying 11 copies of Wp (Tierney et al., 2007). The first part of this thesis examines the importance of Wp copy number for the initiation and/or maintenance of B-cell growth transformation and seeks to determine just how many W copies are needed for efficient transformation *in vitro*.

3.1 Generation of recombinant EBV carrying different numbers of BamHI W

In order to explore the role of BamHI W copy number in B cell growth transformation, a panel of recombinant EBV BACs carrying different numbers of BamHI W (nW) were first generated (Figure 3.1). A wild-type B95.8 EBV-derived BAC (2089) was used as the parental BAC. The 2089 BAC genome contains a GFP-expressing gene, as well as chloramphenicol and hygromycin resistance genes for selection in bacteria and eukaryotic cells, respectively. 2089 DNA was first transformed into *recA*$^{+}$ BJ5183 *E coli* by

electroporation. Transformants were then selected on chloramphenicol agar plates. Due to the active recombinase in the BJ5183 cells, recombination occurred between the BamHI W fragments resulting in loss of some repeats from the 2089 genome in some transformants. This allowed one to generate recombinant EBVs with different numbers of BamHI W copies. BAC DNA was isolated from several clones using alkaline lysis miniprep method, digested with BamHI restriction enzyme and subjected to gel electrophoresis. The restriction profile of BamHI digestion (Figure 3.2A) showed the genome was intact and the only difference seen was in the brightness of the 3kb BamHI W band compared to that from 2089 indicating that some of these clones contained lower numbers of BamHI W copies. The DNA from these clones was then digested with EcoRI and HindIII and subjected to field inversion gel electrophoresis (FIGE). EcoRI and HindIII cut either side of BamHI W region and therefore the digestion profile allows one to determine the number of BamHI W copies in the genome. Figure 3.2B shows the EcoRI and HindIII restriction profile of the selected clones differing only in the size of the band containing the BamHI W repeat region. Recombinant EBVs carrying 0 to 9 copies of BamHI W were selected and DNA of the selected clones transformed into the *recA*$^{-}$ DH10B *E coli* strain to prevent any additional recombination occurring. Transformants were rescreened again and glycerol stocks were made from the selected clones and stored at -80ºC.

To generate virus-producing clones, the BAC DNA was purified and transfected into 293 cells using Lipofectamine 2000 (Invitrogen). Stably transfected clones were selected by growth in hygromycin B and gradually expanded to generate cell lines. For each recombinant, many clones were grown out and tested for their ability to produce virus. For

each recombinant, the best two virus producing clones were selected for further study. To produce virus stocks from these cells, lytic cycle replication was induced by co-transfection of two plasmids, p509 (Hammerschmidt and Sugden, 1988) and pRA (Neuhierl et al. 2002), which express the immediate early transactivator BZLF1 and late viral glycoprotein BALF4 (gp110), respectively. Supernatant from cells induced for 72 hours was harvested, concentrated and purified by density gradient centrifugation and the virus titre quantified by Q-PCR specific for the EBV polymerase gene BALF5. Virus stocks were then stored at -80ºC.

3.2 Infection of resting B cells with recombinant viruses

3.2.1 Viral binding and internalisation into resting B cells

The first set of experiments aimed to examine the very early events following EBV infection of primary resting B cells in terms of virus binding and its subsequent internalisation using recombinant EBV carrying 0 to 7 copies of BamHI W repeat (0W-7W). Fresh primary B cells were isolated from buffy coats by CD19-positive selection (% purity was consistently greater than 90% by FACS analysis) and exposed to purified virus at a multiplicity of infection (m.o.i.) of 50 virus genome copies per cell. To measure the amount of each virus initially bound to the cells, B cells were exposed to purified virus for 3 hours at 4ºC. Unbound virus was washed off and cells harvested for DNA extraction. To measure the amount of each virus that had entered the cells, B cells were exposed to purified virus for 3 hours at 4 ºC, followed by 18 hours incubation at 37 ºC and treatment with chymotrypsin to strip off any virus particles still bound to the cell surface. DNA was extracted from the cell samples and subjected to Q-PCR assay for the EBV polymerase

gene (BALF5) and cellular gene, β2-microglobulin to determine the numbers of virus genomes. Namalwa-BL, containing 2 integrated copies of EBV per diploid cell, was used as a reference line. Figure 3.3 shows the result of Q-PCR assays from one representative B-cell infection experiment measuring the amount of each virus binding (Figure 3.3A) and internalising (Figure 3.3B). In this experiment, all viruses bound with similar efficiency with between 22 (7W) and 35 (6W) virus genomes binding per cell. The number of virus genomes internalised was also similar with between 0.7 and 2.7 genomes per cell. The same experiment was carried out 3 times and in each case, the data showed that viruses carrying different number of BamHI W copies bound and entered B cells with similar efficiency. However, it should be noted that due to differences in the source of blood samples (buffy coats or apheresis cones (cones)), and variation between individual donors, the absolute number of virus genomes bound/internalised per cell differed in each experiment. Therefore, only one representative result is shown here and in future experiments.

3.2.2 EBV gene transcription at early time points following B cell infection

The effect of reducing the number of BamHI W copies on the initiation of EBV gene transcription early after infection was then examined by Q-RT-PCR. Freshly isolated primary B cells were infected with recombinant nW viruses at an m.o.i. of 50. Total RNA was harvested from the cultures of infected cells at 24-hours intervals over 3 days post infection, reverse-transcribed into cDNA and subjected to Q-PCR for EBV latent gene transcripts and the endogenous control GAPDH. The positions of the primers and probe sequences relative to the transcripts are shown in Figure 3.4A. The level of EBV transcripts

were normalised to GAPDH to allow for variations in cDNA input, and results are plotted relative to a suitable reference cell line assigned an arbitrary value of 1. This experiment was carried out 3 times independently and the results from a representative experiment, examining the cells infected with 0W-7W over 3 days post infection are shown in Figure 3.4B. The first two rows of the graphs show that there was no detection of Wp-initiated, BHRF1, EBNA2 and Cp-initiated transcripts in cells infected with 0W or 1W viruses. The third row of the graphs shows that there was low level of transcripts detected in cells infected with 2W virus, while cells infected with 3W-5W virus showed progressively increasing level of transcripts. Cell infected with 6W and 7W viruses showed similar level of transcripts to 5W. Overall, cells infected with 3W-7W viruses showed similar kinetics of transcription; Wp was activated to very high levels within the first 24 hours post infection, followed by a rapid and then gradual decline. BHRF1 transcripts peaked within 24 hours post infection and then rapidly declined, exactly matching the kinetics of Wp-activity. EBNA2 transcripts also showed a pattern with a peak expression within the first 1 or 2 days, with higher copy number virus peaking earlier. Cp was activated at very low levels in the first 24 hours post infection and gradually increased. In contrast, cells infected with the 2W virus showed different kinetics; a lower level of Wp was activated in the first 24 hours, followed by a gradual increase up to 48 hours with the same level remaining at 72 hours. Both BHRF1 and EBNA2 transcripts showed similar patterns, matching the kinetics of Wp activity. Figure 3.5 shows the results from a second similar experiment using 0W-5W viruses with time points at 18 hours, 24 hours and 48 hours post infection. Similar patterns of Wp-, and Cp-initiated and viral gene transcription were seen confirming the previous data. Thus, in conclusion, no viral transcripts were detected in 0W and 1W virus infections;

a low level of transcripts was detected with 2W virus, while the overall level of transcripts increased progressively from 3W to 5W virus. These results indicate that at least 5 copies of Wp are required for optimal activation of Wp and for the subsequent switching to Cp. It should be noted that, in a later B-cell infection experiment (Figure 4.2) using B cells isolated from cones rather than buffy coats, B cells infected with 1W virus had some detectable Wp-initated, EBNA2 and Cp-initiated transcripts but at levels 10 fold lower than 2W virus. However in these later experiments, there was still no detectable transcription from the 0W-infected cells (Figure 4.2).

3.2.3 EBV protein expression at early time points following B cell infection

Two of the earliest markers of viral infection in B cells, EBNA-LP and EBNA2 were monitored by immunofluorescence cell staining. B cells were infected with 0W and 2W-6W at an m.o.i of 50, harvested at day 3 post infection, spotted onto a 10-well slide, fixed and stained for EBNA-LP and EBNA2 using monoclonal antibodies (mAbs) JF186 (1/100) and PE2 (1/200) respectively (Figure 3.6A). EBNA-LP-positive and EBNA2-positive cells (stained red) and the total number of DAPI positive cells (blue) were counted on 4 fields of at least 100 cells to determine the percentage of EBV antigen-expressing cells (Figure 3.6B). In line with the Q-RT-PCR data, no EBNA-LP and EBNA2 were observed in cultures infected with 0W and 1W virus and few cells from the 2W infected culture stained positive for EBNA-LP and EBNA2 (2.3% and 4.9%, respectively). The percentage of EBNA-LP- and EBNA2-positive cells increased by nearly 10 fold with 3W virus (20% and 27.7%, respectively), and there was a further slight increase with 4W virus (26.6% and 33.3%, respectively), while 5W and 6W were similar to each other in having significant

more antigen staining (68.7% and 47.2%, respectively in 5W and 55.7% and 57%, respectively in 6W).

3.2.4 Transformation efficiencies of recombinant viruses

The ultimate test of a virus' B-cell-transforming activity requires the analysis of cultures for cell proliferation leading to successful LCL outgrowth. To quantitate the transformation efficiencies of the recombinant viruses, aliquots of primary B cells were infected with the same panel of recombinant nW viruses (0W-7W) at m.o.i.s of 1, 5, 10, 20, 50 and 100. Infected B cells were then plated out at 10^4 cells/well in 96-well plates , grown with regular feeding, and after 6 weeks the number of wells showing actively growing transformed cell foci were counted. Figure 3.7A shows the results from one such experiment plotted as bar charts. The top two graphs show that the 0W and 1W virus failed to transform B cells at any of the m.o.i. during the 6 weeks post infection. 2W showed a low level of transformation in comparison to 3W-6W; transformation progressively increased from 3W to 5W, while 5W, 6W and 7W showed similar transformation efficiency. Figure 3.7B shows the data from the same experiment plotted as log (m.o.i.) against number of wells transformed, which allows one to determine the m.o.i at which 50% of wells are transformed for each nW virus Figure 3.7C shows that for 2W virus, the m.o.i. for 50% transformation was 44.8; for 3W and 4W, the m.o.i. was approximately 10 fold lower (m.o.i.: 4.4 and 5.0, respectively). For 5W, 6W and 7W, the m.o.i. for 50% transformation (0.04, 1.9 and 0.2, respectively) was at least 30-50 fold lower than for 2W. The high m.o.i. required for 50% transformation seen in 2W virus indicates that the 2W virus was severely impaired for transformation efficiency.

Figure 3.8 shows the results from a similar experiment with 0W to 7W viruses at m.o.i.s of 0.1, 0.5, 1, 5, 10, 20, 50 and 100. As before, no transformation was seen with 0W and 1W virus infections, and a progressively increasing level of transformation was seen with 2W to 5W (optimal transformation efficiency) (Figure 3.8A). Data from the same experiment were plotted as log (m.o.i.) against number of wells transformed (Figure 3.8B). In this particular experiment, the m.o.i. for 50% transformation with 2W was 90.7, which decreased to between 0.2 and 0.5 with the 5W and 7W virus (Figure 3.8C). The overall pattern of results was similar to that observed in the first transformation assay (Figure 3.7). In conclusion, the 2W virus transformed with significantly less efficiency than any of the other W viruses. The 5W, 6W and 7W viruses transformed most efficiently, with 3W and 4W transforming with an intermediate efficiency. This result correlated with the original data from the QRT-PCR assay measurement of Wp- and Cp-initiated transcripts. When we tested virus with higher copy number of BamHI W (data not shown), the transformation efficiencies for 8W, 9W and 11W (2089), were very similar to 5W, 6W and 7W, indicating that 5 BamHI W copies are sufficient for maximum transformation efficiency. Taking together the data from Q-RT-PCR, IF staining and transformation assays, we conclude that at least five copies of the BamHI W fragment (i.e. five copies of Wp) are required for optimal promoter activation and efficient B cell transformation *in vitro*.

3.2.5 EBNA-LP expression in established LCLs

B cells transformed by each nW virus were cultured and expanded for several weeks to establish LCLs. Figure 3.9 shows the western blot detecting the EBNA-LP expression in each established LCL. Unexpectedly, EBNA-LP expressed from 2W-derived LCLs showed

sizes equivalent to 2 and 3 repeat EBNA-LP; EBNA-LP from 3W-derived LCLs showed the sizes equivalent to 3 and 5 repeat EBNA-LP and EBNA-LP from 4W-derived LCLs showed the sizes equivalent to 4, 5 and 6 repeat EBNA-LP. Whereas the sizes of the EBNA-LP expressed from 5W-, 6W- and 7W-derived LCLs correlated to the number of BamHI W copies in the viruses (5, 6 and 7 repeat EBNA-LP, respectively).

3.3 Infection of pre-activated B cells with recombinant viruses

In order to determine whether infection of pre-activated human B cells might overcome the failure of 0W and the impairment of 2W virus in activation of viral gene transcription in resting primary B cells, we repeated the previous experiments in cycling B blasts. B blasts were generated from resting primary B cells by co-cultivation on CD40 ligand-expressing feeder cells with medium containing IL4 for 14 days, and then removed from the feeders and IL4 containing medium immediately before infection with 0W, 2W, 3W and 5W viruses. In parallel, infection of BL41, an EBV-negative Burkitt lymphoma cell line was also analysed. This experiment was carried out 3 times independently and Figure 3.10 shows Q-RT-PCR analysis from one representative experiment comparing Wp-initiated, Cp-initiated and EBNA2 transcription on day 2 post infection. As shown previously, resting B-cell infection with 0W virus showed no Wp and Cp activities and no EBNA2 transcription was detected. Infection with 2W virus resulted in very low level of Wp and Cp activation and EBNA2 transcription while 3W and 5W virus gave high levels of Wp-initiated, EBNA2, and Cp-initiated transcription. Overall the same pattern and similar levels of gene transcription were seen in infected B blasts and BL41 cells as in resting B cells, indicating that the cellular environment of pre-activated cycling B cells could not

overcome the failure of 0W and the impairment of 2W virus in initiating the gene transcription associated with the growth transformation program.

Transformation assays using B blasts made as above were carried out to determine whether the transformation efficiency of 2W virus could be enhanced in pre-activated cycling B cells. However, no transformation was ever observed using B blasts made in this way (data not shown). Therefore, alternative experiments were then carried out using soluble CD40 ligand and IL4 in order to determine whether this method of B cell activation could enhance the transformation ability of the impaired 2W virus. Resting primary B cells were infected with either the 2W or 5W virus at m.o.i.s. of 0.1, 1, 5, 10, 25, 50, 100 in duplicate. One set of infected B cells was then plated in soluble CD40 ligand and IL4 containing medium at 10^4 cells/well in 96-well plates; the other set of infected B cells was plated in regular medium at 10^4 cells/well in 96-well plates. Thereafter all cultures were fed weekly using medium without CD 40 ligand (CD40L) and IL4, therefore only the virus-transformed cells could grow out to become LCLs. After 6 weeks, all wells with actively growing transformed cell foci were counted and the results plotted against m.o.i. Figure 3.11A shows the result from the B cell infection without CD40L/IL4 and Figure 3.11B shows the result from the parallel assay with CD40L/IL4. In this experiment, 100% of transformation was seen down to 25 m.o.i. in 2W infection with or without CD40L/IL4, and down to 5 or 10 m.o.i. in 5W infection with or without CD40L/IL4. Data from the same experiment were plotted as log (m.o.i.) against number of wells transformed (Figure 3.11C). Figure 3.11D shows that for 2W virus without CD40L/IL4, the m.o.i. for 50% transformation was 11.5, which is similar to the m.o.i for 2W virus with CD40L/IL4 (m.o.i:

10.4); the m.o.i. for 50% transformation for 5W with or without CD40L/IL4 were also very similar (0.5 and 0.6, respectively). Therefore, we concluded that there is no significant difference in B cell transformation with or without CD40L/IL4 in either 2W or 5W.

3.4 Discussion

Epstein-Barr virus possesses a unique set of latent genes whose constitutive expression in B cells leads to cell growth transformation. This growth transforming program is initiated through the activation of a promoter, Wp, which is located within each BamHI W repeat of the EBV genome. Early work on Wp regulation from this laboratory using *in vitro* reporter assays identified the presence of three regulatory regions, UAS1, UAS2 and UAS3 and identified the transcription factor binding sites in UAS1 and UAS2 (Bell et al., 1998, Kirby et al., 2000, Tierney et al., 2007). In order to understand the Wp regulation in the more complex setting of the natural virus genome, Tierney et al. subsequently generated a series of recombinant viruses carrying different mutations in the Wp UAS1 and UAS2 regulatory regions (Tierney et al., 2007). However, because of the complication of Wp's existence in multiple copies, it was difficult to make mutations in every Wp copy. With previous studies showing that viruses carrying only two copies of Wp transformed B cells *in vitro* (Kempkes et al., 1995b, Yoo et al., 1997), a recombinant EBV BAC with only two BamHI W copies was generated and used as the parental construct to generate mutant recombinant EBVs in which both copies of W contained the desired mutations (Tierney et al., 2007).

The work in this chapter was prompted by the observation from the above work that 2W wild-type virus, though capable of transformation, was less active in this respect than the

prototype EBV recombinant 2089, which had 11 wild-type W copies (Tierney et al., 2007). In the first section of this work, we tried to understand to what extent the number of W copies is important in initiation of B-cell growth transformation and/or maintenance of the transformed phenotype and how many Wp copies are required for efficient transformation *in vitro*.

In order to understand the role of Wp copy number in B-cell growth transformation in the context of the virus, a panel of EBV recombinant viruses with different numbers of BamHI W fragments was initially generated using the B95.8-derived BAC system. The amount of virus produced from recombinant BAC containing 293 cells following BZLF1 induction of the lytic cycle was found to vary significantly between different 293 clones. In addition, variation also occurred between virus batches produced from the same 293 clones at different times. Therefore, to be able to compare these nW viruses, it was important to equalise the amount of virus in stocks made from each virus induction and to ensure the purity of the virus stocks. Measuring the green Raji cells (GFP-positive cells) after infection of Raji cells with the virus stocks, called the “green Raji units” assay has been used to determine the infectious virion content in some studies (Dirmeier et al., 2003, Neuhierl et al., 2002). However, we have observed that recombinant viruses tend to gradually lose GFP expression after passage in culture, and produce infectious virion without GFP. Therefore, using “green Raji units” may underestimate the virion content. A second method using Q-PCR amplification of the EBV polymerase gene (BALF5) to determine virion content has been reported (Shannon-Lowe et al., 2005). However, using Q-PCR to determine virion content may overestimate by detecting free DNA in the virus

prep. Therefore, intact whole virus particles were purified away from empty capsids, microvesicles and cell debris using Optiprep (Axis Shield) self-generated gradient centrifugation (Moller-Larsen and Christensen, 1998) to ensure the purity of the virus stocks before titrating using Q-PCR.

B cells are another key component in this work. As we had no constant source of tonsils from which to isolate B cells, we instead used buffy coat as the source. Due to the limited amount of B cells we could isolate from the buffy coats, it was impossible to characterise the viruses in all aspects (binding, internalisation, viral RNA transcription, protein expression and transformation) in a single experiment. Additionally, because of changes at the Blood Transfusion Service in Birmingham part way through the work, the source of B cells changed from buffy coats to apheresis cones (cones). We observed that the cones which were obtained earlier than buffy coats relative to the time of bleeding, produced better yields with greater cell viability for infection experiments. As a result of this difference in the source of blood samples (buffy coats vs. cones) and of natural variation between B cells preparation from individual donors, the absolute values for virus binding, internalisation, transcription, protein expression and transformation vary between experiments but the overall patterns of results (comparing viruses with different copies) remained the same. Therefore, the results from a representative single experiment are shown throughout this part of the work.

First, the nW virus preparations were added at equal m.o.i for virus binding and the subsequent internalisation assays (Figure 3.3). In the example shown, infection with m.o.i

of 50, each nW virus gave a similar number of virus genomes (between 22 and 35 per cell) bound and a similar number (between 0.7 and 2.7 per cell) internalised into B cells. Thus, approximately 10% of the virus bound to the cell surface internalised, which is a similar proportion to that previously reported (Shannon-Lowe et al., 2005). This experiment was repeated several times (Figure 3.3 and data not shown).While the absolute values of virus binding and internalisation varied between experiments, we generally observed a similar level of binding and internalisation for all the viruses within a single experiment. Therefore, we believe that there was no significant difference seen between any of the nW viruses in their ability to bind and deliver their genome into B cells. With this as background, we could then directly compare the viruses for gene expression post infection and subsequently for transforming ability.

For this purpose, we focused on two sets of assays looking at early transcription and antigen expression (during the first 72 hours post infection) and long-term transformation (after 6 weeks culture). The early transcriptional events in B-cell infection were first monitored by Q-RT-PCR. Wp-initiated, Cp-initiated and EBNA2 transcripts were quantitated by high sensitivity Q-RT-PCR assays that were capable of detecting a single expressing cell in a background of 10^5 negative cells (Bell et al., 2006). Transcripts encoding BHRF1, recently described as a latent protein and strongly related to Wp activity (Kelly et al., 2009), were also monitored by Q-RT-PCR assays using an LCL generated with a lytic cycle-deficient BZLF1-knocked out virus (BZKO) as a standard. The primers for all the Q-RT-PCR assays were designed to go across splices (e.g. for EBNA2, the

reverse primer and probe are in the YH exon but the forward primer spans the Y2/YH junction), thus determining the RNA specificity of the assays.

From the results of the Q-RT-PCR assays, we saw a correlation between the level of Wp-initiated transcripts and the numbers of Wp copies. There was a trend for increasing Wp activity going from 1W to 5W, suggesting that most (if not all) of the Wp promoters in these viruses were functional immediately post infection. However, it should be noted this does not apply to viruses carrying more than 5 copies of Wp. Beyond 5W, viruses showed similar levels of Wp-initiated transcripts, implying they had reached the maximum threshold level of Wp usage. This phenomenon may be down to the transcriptional interference from upstream Wp-initiated transcripts affecting downstream Wp usage, in a manner like that proposed for Cp interfering with Wp usage later in the infection process (Puglielli et al., 1996, Yoo et al., 1997, Puglielli et al., 1997).

BHRF1 was originally recognised as a lytic gene but in recent studies, it has been reported that BHRF1 transcripts are expressed very early post infection. Furthermore, BHRF1 gene-deleted virus had lower transforming ability at low m.o.i., suggesting that BHRF1 protein (a Bcl-2 homologue) was protecting early infected B cells from apoptosis (Altmann and Hammerschmidt, 2005). More recently, it was shown that there was a strong connection between Wp activity and BHRF1 transcription, and that BHRF1 expression is also a feature of all *in vitro* transformed cells (Kelly et al., 2009). The results in Figure 3.4 and Figure 3.5 showed a burst of BHRF1 transcription immediately post infection followed by rapid decline, which matched the kinetics of Wp activity as previously reported (Kelly et al.,

2009). Furthermore, viruses with more copies of Wp also showed more BHRF1 transcripts, supporting that view that Wp is driving BHRF1 expression in early infection.

EBNA2 is encoded by Wp-initiated transcripts immediately post infection (Alfieri et al., 1991). Figure 3.4 and Figure 3.5 showed that the kinetics of EBNA2 transcription within the first 72 hours post infection were similar to the kinetics of Wp activity, viruses with higher number of Wp copies showing higher EBNA2 transcripts. EBNA-LP is also encoded by Wp-initiated transcripts and EBNA2 and EBNA-LP are the first two viral antigens expressed immediately post infection. Expression of these two early viral antigens at the single cell level was detected by immunofluorescence cell staining and recorded as the percentage of antigen-positive cells. Consistent with the Q-RT-PCR data, no EBNA-LP and EBNA2 expression was detected in cells infected with 0W virus and progressively increasing level of EBNA-LP and EBNA2 were expressed from 2W to 5W viruses; whereas virus carrying more than 5 copies of Wp showed no further increase.

EBNA2, acting either alone or in cooperation with EBNA-LP, then serves to activate the alternative EBNA promoter Cp (Yoo and Speck, 2000, Schlager et al., 1996, Jin and Speck, 1992, Harada and Kieff, 1997), and this leads to the broadening of viral antigen expression. Overall, our two experiments (Figure 3.7 and Figure 3.8) indeed showed that the level of Wp activity declined after 24 hours post infection as the level of Cp activity increased. However, looking closely, with 3W to 7W viruses, Wp activity peaked within the first 24 hours post infection and was followed by a rapid and then more gradual decline, while Cp activity was induced later, becoming apparent by 48 to 72 hours post infection. This

promoter-switching phenomenon matched previous observations (Schlager et al., 1996, Woisetschlaeger et al., 1991, Woisetschlaeger et al., 1990). However, with 2W viruses, different kinetics were observed; the level of Wp-initiated transcripts was less than 20% of that in 5W virus. With the low Wp activity in 2W virus, the level of Cp induced was also very low and the promoter switching was not apparent within the time scale of the experiment.

In the case of 0W, the deletion of all Wp copies means there is no Wp-initiated EBNA2, EBNA-LP or EBNA1 products which could potentially activate Cp (Harada and Kieff, 1997, Jin and Speck, 1992, Nilsson et al., 2001, Schlager et al., 1996, Yoo and Speck, 2000). As might be predicted, we found that in the absence of Wp, Cp was not activated. Therefore, unlike Wp, Cp is dependent on viral factors (possibly EBNA2, EBNA-LP and/or EBNA1) for its activation. Since Cp reporter constructs are active when introduced into cycling EBV-negative BL cell lines, we wondered whether the pre-activation of normal resting B cells into cycle with mitogenic signal (CD40L/IL4) might allow Cp to become active in the 0W to 2W viruses (Figure 3.10). In fact, Cp activity remained silent in cycling B cells infected with 0W virus, and was not increased with 1W to 2W viruses, suggesting that Cp is absolutely dependent on the viral factors.

Note that in the case of the 1W virus, we failed to detect any Wp or Cp activity in B cells isolated from buffy coats; however in more recent experiments, with B cells isolated from cones, we could detect extremely low levels of Wp and Cp activity (about 100 fold less than 5W virus) and an even lower level of EBNA2 transcription (more than 100 fold less

than 5W virus). Further work needs to be carried out to address the issue of EBNA-LP and EBNA2 protein expression in such experiments (immunofluorescence staining and Western Blotting). But clearly, the 1W virus is significantly more impaired than the 2W virus at these early times post infection.

Transformation assays were carried out in separate experiments due to the large amount of virus and B cells required to set up one whole microtest plate (96 replicate cultures) per nW virus per virus dose. However, in general, the transforming ability of the different nW viruses was in line with their early transcriptional activities. Thus, 5W-7W viruses transformed most efficiently (similar to 2089, data not shown), with 3W and 4W transforming with an intermediate efficiency (10 fold less than 5W virus), whereas 2W transformed with very low efficiency (approximately 10 fold less than 3W) and no transformation was ever seen with 0W and 1W (Figure 3.7 and Figure 3.8). Taken together, our data showed that, between 0W and 5W, viruses with more Wp copies had higher levels of Wp activity, produced more EBNA-LP and EBNA2 protein and eventually transformed B cells more efficiently. Increasing Wp copy number beyond five brought no further increase in early latent gene expression or in transforming ability.

As an additional check, LCLs derived from nW virus infections were further analysed by Western Blotting. The majority of the EBNA-LP species from the nW LCLS reflected the number of BamHI W copies present in the viruses. For example, in Figure 3.9, an EBNA-LP protein of expected size (ie. 7 W1W2 repeat domains) is the most abundant species in a 7W-infected LCL, likewise for 6W, 5W, 4W and 3W transformed LCLs. This is consistent

with the view that in established LCLs, at least when made with viruses up to 7W copies, the dominant EBNA-LP transcript contains all the available W1W2 repeat exons. This transcript may of course be initiated at Cp, but a more attractive idea is that it is initiated from the most 5' Wp copy. This would fit with what the earlier studies of Yoo et al. showed (Yoo et al., 1997, Yoo et al., 2002), and also with the notion that a small number of Wp transcripts remain present in LCLs and may be initiated from the most 5' Wp copy, the other copies having been methylated by this time (Hutchings et al., 2006, Tierney et al., 2000b).

However note that in several cases, some larger than expected EBNA-LP proteins were seen in the gel. Furthermore an unexpectedly larger EBNA-LP (equal to 3 W1W2 repeat domain) was detected in the 2W-LCL, despite the fact that during the first few days post infection with 2W virus, only an EBNA-LP of the expected size was detected (see later, Figure 4.12B). It should be noted that it took several weeks to expand the transformed foci from microtest plate wells to flasks before there were sufficient cells for protein analysis. Thus it is conceivable that virus recombination had occurred within the culture to generate a new virus with more W copies, and if this had a growth advantage, the cells carrying it could have eventually taken over the culture.

However, a second possibility is that this larger than expected EBNA-LP protein is generated via trans-splicing of the RNA. Thus, during splicing of the primary Cp/Wp EBNA-LP transcript, the introns are removed and the coding W1, W2, Y1 and Y2 exons are joined together to make the mature mRNA. Normally exons can only be joined together

if they are part of the same primary transcript. However in trans-splicing, an exon from one RNA molecule can be joined to the exon of a second RNA molecule (Rickman et al., 2009, Gingeras, 2009). In this case, W1 and W2 exons from one EBNA-LP transcript could be joined to the W1 or W2 exons of a second LP transcript. In theory, this means that a 2W LCL could make 3 or 4 repeat EBNA-LP proteins if two RNAs were trans-spliced.

EBV has acquired multiple copies of the BamHI W repeat sequence during evolution. The present data suggest that viruses with such multiple repeats have an advantage, namely they are more capable of driving EBNA-LP/EBNA2 expression immediately post infection because at least below 5 Wp copies, all Wp copies are active. Indeed, the ladder of EBNA-LP species seen in the early stages of EBV supports the notion that transcription initiates from multiple copies of Wp (Finke et al., 1987, Rooney et al., 1989). Previous studies have shown that virus isolated from infected individuals by B cell transformation contained 3 to 12 copies of Wp (Allan and Rowe, 1989). Since we now know that this will select for viruses with ≥ 5 Wp copies, these *in vitro* isolates may not reflect the true range of viruses *in vivo*. It would be interesting to determine how many BamHI W repeats are found in naturally occurring virus genomes in *ex vivo* assays. However, with current techniques this is not possible since it would require large amount of viral DNA. Another possible approach is to study the monoclonal virus genomes captured in EBV positive malignancies. In fact, we know that BL lines carry viruses with greater than 3 W copies (Allan and Rowe, 1989), but again it might be argued that B cell transforming ability is a prerequisite for BL pathogenesis. Therefore, the virus isolates from nasopharyngeal carcinoma (NPC) cells

where B cell transforming ability is irrelevant may reflect the situation in natural viruses more closely.

The work in this part of the thesis also has implication for all studies using recombinant viruses where B cell transformation is the read-out. Clearly recombinant viruses are very important tools which allow one to look at individual gene function in the context of the whole EBV genome. However, our experiment shows the ease with which the EBV genome lost copies of BamHI W during the recombination procedure in bacteria. Therefore, it is important that in any further studies with recombinant viruses carrying specific mutation/deletion at any site in the genome are carefully controlled for the number of BamHI W copies in the mutated virus. At least five copies of BamHI W fragments have to be present and ideally when comparing different mutants for transforming ability, they should have the exactly same number of W copies.

Chapter 4. Rescuing the growth transformation of impaired nW viruses

From the results shown in Chapter 3, it is clear that the number of BamHI W copies present in the genome has an effect on EBV induced transformation of B cells. Virus with BamHI W deleted or with only one copy of BamHI W failed to transform B cells; virus with two copies of BamHI W showed severely impaired transformation ability, while viruses with three, four or five copies of BamHI W showed progressively increasing transforming ability. However, increasing the BamHI W copy number beyond 5 had no further effect. Therefore at least 5 copies of the BamHI W (i.e. five copies of Wp) are required for promoter activation and efficient B cell transformation. To some extent this may reflect the fact that, with Wp copies reduced in number, Wp transcription is limiting in freshly-infected B cells. However another consequence of reducing BamHI W copy number is to reduce the size of one of the key latent proteins, EBNA-LP, whose repeat sequence is encoded by the W1 and W2 exons within each BamHI W repeat; therefore the 2W virus can only make an EBNA-LP with two copies of the W1W2 repeat domain. In order to distinguish between the effect of reducing Wp copy number itself and the knock-on effects from reducing EBNA-LP size, different strategies were used to try and rescue the impaired transformation ability of the 2W virus.

4.1 Rescue with helper virus

One potential strategy to rescue the impaired transformation with 0W, 1W and 2W viruses was to use helper virus coinfection. Two viruses, an EBNA2 gene knockout (called E2KO)

and a LMP1 gene knockout (called LMP1KO) were kindly provided by Wolfgang Hammerschmidt. E2KO virus was made from the parental 2089 recombinant virus by deleting the entire coding sequences of EBNA2 (Altmann and Hammerschmidt, 2005) and was not able to transform primary B cells. The other helper virus, LMP1KO virus was made from the same parental 2089 virus with the deletion of the entire open reading frame of LMP1. LMP1KO virus was able to generate proliferating B-cell clones, but was only able to sustain their growth with the aid of fibroblast feeder cell layers (Dirmeier et al., 2003).

4.1.1 B cell infection with E2KO and LMP1KO helper virus

Initially, Western blot analysis was carried out to characterise the E2KO and LMP1KO virus gene expression in resting B cells. Resting primary B cells were infected with E2KO and LMP1KO virus separately at an m.o.i of 50 and $1x10^7$ infected cells were harvested at 1, 3 and 7 days post infection. Protein samples were extracted from the cells and analysed by Western blotting using the monoclonal antibodies shown in Table 2.6. This experiment was carried out 2 times and Figure 4.1 shows the results from one representative experiment. Firstly, no EBNA2 was ever detected in E2KO-infected B cells, confirming that EBNA2 was totally knocked-out in the E2KO virus. In the same cells, EBNA-LP was detected as a ladder of bands by one day post infection. Very low levels of BHRF1 were also detected by one day post infection and gradually increased during the time course. EBNA1, EBNA3A and EBNA3C proteins were also detected in E2KO-infected B cells by one day post infection. Surprisingly, LMP1 was also detected starting at three days post infection with higher levels detected by seven days post infection.

When the LMP1KO virus was used to infect resting B cells *in vitro*, no LMP1 was ever detected, confirming that LMP1 was successfully knocked-out in the LMP1KO virus. Considerable levels of EBNA2 and EBNA-LP (a ladder of bands) were detected by one day post infection in LMP1KO-infected B cells. Although no BHRF1 was detected, it should be noted that the antibody for BHRF1 was poor and by a more sensitive Q-RT-PCR assay, very low levels of BHRF1 transcripts could be detected (Figure 4.2). EBNA1, EBNA3A and EBNA3C protein could be detected by three days post infection. Western blotting of a second repeat experiment with the E2KO and LMP1KO viruses gave similar results (data not shown).

In conclusion, E2KO virus expressed a sufficient amount of large EBNA-LP and all the other latent proteins tested except EBNA2, while LMP1KO virus expressed sufficient amount of large EBNA-LP and EBNA2, along with the other latent proteins tested except for LMP1 and maybe BHRF1. Therefore, for our purposes, these two non-transforming viruses were very useful as helper viruses because co-infection with the impaired nW viruses allowed us to assess the importance of EBNA-LP (with E2KO) and EBNA-LP plus EBNA2 (LMP1KO) in B cell transformation.

4.1.2 Coinfection of recombinant nW viruses with helper viruses in resting B cells

A series of Q-RT-PCR assays were then carried out to track the initial Wp- and Cp-initiated transcription and BHRF1 and EBNA2 transcripts. The fresh B cells used in this experiment were isolated from cones, infected with recombinant viruses (1W and 2W) with or without

helper viruses (E2KO and LMP1KO) and RNA isolated from infected cells harvested at 1, 2 and 3 days post infection. This experiment was carried out 2 times independently and the results from one representative experiment are shown in Figure 4.2. The kinetics of transcription in the 2W virus from this experiment were very similar to that previously observed (Figure 3.4), however interestingly, unlike the previous experiments, B cells infected with 1W in this experiment showed some Wp-initiated, BHRF1, EBNA2 and Cp-initiated transcripts. This is most likely due to superior quality of B cells isolated from cones compared to buffy coats. However, the transcription seen in the 1W virus was 10 fold lower than transcription in the impaired 2W virus.

Very high Wp-initiated and BHRF1 transcripts were detected in cells infected with E2KO virus alone, and as expected, no EBNA2 transcripts were detected. Interestingly, the level of Cp activity in E2KO was low and similar to the level in 2W virus. High levels of Wp-initiated transcripts were also detected in cells infected with LMP1KO virus alone, however, surprisingly, very low levels of BHRF1 transcripts were detected. In addition, high levels of Cp-initated and EBNA2 transcripts were detected in LMP1KO virus with kinetics similar to those seen with 5W virus (Figure 3.4).

Due to the dominant transcription seen from E2KO and LMP1KO virus, it is difficult to distinguish the transcription from the nW virus and the helper virus. However, in this experiment, no increase in the level of EBNA2 transcripts was observed in coinfection with 2W and E2KO viruses in comparison to 2W virus alone. Therefore, we then carried out

transformation assays to directly quantitate the transformation efficiencies with or without helper viruses.

Primary B cells were infected with the 0W, 1W or 2W viruses at m.o.i.s of 1, 5, 10, 20, 50 and 100. In parallel, B cells were co-infected with the same nW virus at the same panel of m.o.i.s and either the E2KO or LMP1KO helper virus at 50 m.o.i. Controls of B cells infected with E2KO or LMP1KO virus alone (50 m.o.i.) were also set up. The infected B cells were then plated out at 10^4 cells/well in 96-well plates, grown with regular feeding, and scored after 6 weeks for wells showing transformed foci as previously described. Figure 4.3A shows the results from one such experiment plotted as bar charts. The first row of graphs shows that the 0W virus failed to transform B cells at any of the m.o.i. with or without helper virus during the 6 weeks post infection. The second row of graphs shows that 1W virus alone or 1W plus E2KO helper virus failed to transform B cells at any m.o.i. However, most interestingly, when LMP1KO virus was used as a helper virus, 21 out of 96 wells were transformed with 1W virus at 100 m.o.i. (N.B. infection of 1W in this experiment was only carried out at 100 m.o.i). The third row of graphs shows that the efficiency of transformation with 2W virus was marginally increased by co-infection with the E2KO helper virus and significantly increased by co-infection with the LMP1KO helper virus. Figure 4.3B shows the same data plotted as a graph of log (m.o.i.) against the number of wells transformed, which allowed one to determine the m.o.i.s for 50% transformation. The m.o.i for 50% transformation in 2W was 124.4, which decreased to 20.3 with the E2KO helper virus and further decreased to 3.3 with the LMP1KO helper virus. These data show that use of the E2KO helper virus slightly improved transformation with the 2W virus,

but did not aid the 1W virus. However, the LMP1KO virus provided significant help to the 2W virus and was even able to provide sufficient help to get transformation with a high m.o.i. of 1W virus.

4.2 Repair of 2W virus with a 7 repeat EBNA-LP expressing cassette

Considerable levels of improvement in transformation efficiency of the impaired nW viruses were observed in the previous experiment using E2KO and LMP1KO helper viruses. However, it is difficult for us to conclude that the effects were simply from the EBNA-LP (E2KO) or EBNA-LP plus EBNA2 (LMP1KO) alone, because of the additional latent antigens expressed in these viruses (Figure 4.1). Therefore, we developed a second strategy to rescue the impaired nW virus by creating the repaired viruses, 2W-LP and 2W-EBNA2.

4.2.1 Construction of repaired virus, 2W-LP

2W-LP virus was created by introducing a cDNA encoding a 7 repeat EBNA-LP protein into the 2W virus genome using the BAC cloning system (Delecluse and Hammerschmidt, 2000). Figures 4.4 - 4.6 show this cloning strategy in three parts, and the data validating the construction at each step.

In the first step (Figure 4.4A), plasmid pW3.1LP (Speck et al. 1986) containing EBNA-LP cDNA with seven BamHI W repeats was digested with EcoRI. The 1644bp fragment of EBNA-LP cDNA was purified from a 0.8% agarose gel and ligated into an EcoRI-digested pIRES-GFP vector (Invitrogen) to generate the plasmid, pIRES-LP-GFP. Figure 4.4B

shows HindIII and PciI digests of pIRES-LP-GFP, which gave bands of the predicted sizes confirming that the construction of the plasmid was correct.

In the second part (Figure 4.5A), PCR with primers containing HindIII and PciI restriction sites was used to amplify the SV40-promoter and hygromycin resistance gene from 2089. The PCR product was then purified and digested with HindIII and PciI before being ligated into a HindIII/PciI-digested pCP16 vector, containing a tetracycline resistance cassette to generate a plasmid called pCP16-HygR. Figure 4.5B shows EcoRI/HindIII digests of pCP16-HygR, which gave bands of the predicted sizes confirming that the construction of the plasmid was correct.

In the third and final part (Figure 4.6A), to generate the LP-shuttle vector, pCP16-HygR plasmid was digested with SmaI and PciI and the 4016bp fragment containing the tetracycline and hygromycin resistance cassettes was gel purified, blunt-ended with Klenow polymerase and ligated into pIRES-LP-GFP vector, which had been linearised with AflII and blunt-ended with Klenow. Figure 4.6B shows XbaI, HindIII/XbaI and FspI/HindIII digests of the LP-shuttle vector, which gave bands of the predicted sizes confirming that the construction of the plasmid was correct.

To confirm the LP-shuttle vector was capable of expressing EBNA-LP, the EBV-negative Burkitt's lymphoma-derived cell line DG75 was transiently transfected with 10µg of LP shuttle vector by electroporation and EBNA-LP expression detected by immunofluorescence staining. Transfected cells were harvested at 24 hours post infection

and stained for EBNA-LP using JF186 antibody. Representative EBNA-LP stains (red) and DAPI counterstains (blue) of the same fields are shown in Figure 4.7A. To also confirm that the EBNA-LP expressed from the LP-shuttle vector was the correct size, protein was extracted from the cells from the same transfection experiment, and subjected to Western blotting. EBNA-LP was detected using JF186 and the size confirmed as 66kDa equivalent to 7 copies of the EBNA-LP repeat (Figure 4.7B).

After confirmation of the integrity of the LP-shuttle vector, homologous recombination was used to introduce the EBNA-LP cDNA into the 2W BAC genome with the hygromycin resistance gene and CMV promoter region acting as the homologous regions on either sides of the cDNA (Figure 4.8). The LP-shuttle vector was linearised by digestion with FspI which cuts at a single site in the vector, outside of the EBNA-LP and homologous regions. The linearised DNA was then transformed into rec A^+ BJ5183 *E coli*. containing the 2W BAC plasmid. Recombinants were selected on chloramphenicol/tetracycline agar plates and were screened by BamHI restriction enzyme digestion. A correct clone was identified and the BAC DNA was then transferred into a rec A^- DH10B *E coli*. strain for propagation and maintenance. The BAC DNA was then re-checked by restriction enzyme digestion with EcoRI and HindIII in addition to BamHI. Figure 4.9 shows these restriction profiles of BamHI, EcoRI and HindIII digests in comparison to the parental 2W BAC, which confirmed the construction of 2W-LP BAC was correct. The complete 2W-LP BAC was then transfected into 293 cells and stably transfected clones were selected with hygromycin B, expanded and screened for virus production as previously described.

4.2.2 Infection of resting B cells with 2W-LP repaired virus

To analyse the effect of additional expression of EBNA-LP on infection of primary B cells, a series of binding assays, transcription assays, IF cell staining assays and transformation assays were then carried out comparing the 2W-LP virus with the 2W, 3W and 5W viruses. This experiment was carried out 3 times independently and Figure 4.10 shows the result of Q-PCR assays from one representative experiment measuring the amount of each virus initially bound to the cells and the result demonstrated that all the four viruses bound to B cells with equal efficiency.

Next, a series of Q-RT-PCR assays (Figure 4.11) was then carried out to track the Wp-initiated, BHRF1, EBNA2 and Cp-initiated transcripts from the same B-cell infection experiment as the binding assay. It should be noted that one of the primers for detecting Wp-initated transcripts is in W0, therefore, only Wp-initiated transcripts (not the inserted EBNA-LP cDNA) can be detected. Interestingly, overall, the levels of Wp-initiated, BHRF1, EBNA2 and Cp-initiated transcripts were significantly enhanced nearly 10 fold, with the 2W-LP relative to the 2W parental virus. The kinetics of the transcription in 2W-LP virus were similar to 3W and 5W viruses, however, the levels of transcriptions were much lower (more than 2 fold lower than 3W virus; about 5 fold less than 5W virus).

EBNA-LP and EBNA2 protein expression was monitored by immunofluorescence cell staining. B cells infected with the 2W, 2W-LP and 5W viruses at an m.o.i of 50 were harvested at day 3 post infection and stained for EBNA-LP and EBNA2 (Figure 4.12 A). The number of antigen positive cells (red) and the total number of DAPI stained cells (blue)

were counted on 4 fields of at least 100 cells to determine the percentage of EBV antigen-expressing cells (Figure 4.12B). In the 2W-infected cells, only 10.9% of the cells were EBNA-LP positive, whereas in the 2W-LP infected cells, 26.7% of the cells were EBNA-LP positive, which is similar to the percentage in 5W-infected cells (28.2%). In the 2W-infected cells, only 5.2% of the cells were EBNA2 positive, whereas in the 2W-LP infected cells, 15.5% of the cells were EBNA2 positive, which is lower than the percentage in 5W-infected cells (26.6%). These results suggested that the increased level of EBNA-LP is likely due to the expression from the EBNA-LP cDNA introduced into the repaired virus and more importantly, the increased levels of the antigen expression in the 2W-LP virus correlate to the increased levels of transcription seen in the Q-RT-PCR data.

To further analyse the expression of EBNA-LP and EBNA2, we then carried out western blotting to detect these two proteins in 2W and 2W-LP infected B cells at 3 days post infection. EBNA-LP equal to the size of 2 copies of EBNA-LP was detected in 2W infected cells (Figure 4.12C). A higher level of EBNA-LP was detected in 2W-LP infected cells, but surprisingly its size was not what we expected. The majority of the EBNA-LP was a size corresponding to protein containing 2 repeats, while a low level of EBNA-LP corresponding to protein containing 3 or 4 repeats. In addition, a higher level of EBNA-2 was also detected in 2W-LP infected cells compared to 2W virus (data not shown). Taken together, observation from IF staining matched the data from the western blotting; more EBNA-LP and EBNA2 were expressed in 2W-LP infected cells than in 2W infected cells.

To directly quantitate the transformation efficiency of the 2W-LP virus compared to the 2W parental virus, transformation assays were carried out as previously described. This experiment was carried out 3 times independently and Figure 4.13A shows the results from one representative experiment plotted as bar charts. The relative transformation efficiencies of 2W and 5W were similar to those previously observed (Figure 3.7A and Figure 3.8A). Interestingly, 2W-LP virus showed higher levels of transformation than 2W virus, however, the levels were still lower than 3W and 5W viruses. When the m.o.i. for 50% transformation was calculated (Figure 4.13B and C), 2W-LP had an m.o.i. of 29.7 compared to 44.8 for 2W, representing less than 2 fold improvement in transformation efficiency. However, this was still well below the transformation efficiency of 3W (4.4 m.o.i) or 5W (0.04 m.o.i.).

Taken together, these results indicate that expression of EBNA-LP from another promoter within the virus genome enhances Wp/Cp-initiated transcription, latent protein expression and transformation efficiency but did not to restore these to anywhere near wild type levels.

4.3 Repair of 2W virus with an EBNA2 expressing cassette

From the previous data, we concluded that producing a truncated EBNA-LP with only 2 copies of the W1W2 repeat is unlikely to be the sole reason of the defect in 2W virus. It is also likely that in 2W virus, the 2 copies of Wp are insufficient to achieve optimal Wp-initiated transcription in freshly-infected resting B cells and therefore insufficient to produce enough EBNA2 to activate the Cp and LMP promoters and push the resting B cell

into cell cycle. To further address this issue, another repaired virus, 2W-EBNA2 was created.

4.3.1 Construction of repaired virus, 2W-EBNA2

2W-EBNA2 was made by inserting EBNA2 cDNA driven by CMV promoter into the 2W BAC genome. To generate this virus, the EBNA2-shuttle vector was first constructed by replacing the EBNA-LP cDNA in the previously described LP-shuttle vector with an EBNA2 encoding cDNA (Figure 4.14A). Plasmid pSG5-EBNA2 (Cohen et al., 1992) containing the entire EBNA2-coding region of EBV strain W91, was digested with AvrII and BglII, and the 2542bp fragment containing the EBNA2 cDNA and the β globin intron from pSG5 was gel purified from a 0.8% agarose gel. The LP-shuttle vector was digested with NheI and BamHI (which create compatible overhangs for ligation to AvrII and BglII digested DNA ends, respectively) and subjected to an agarose gel electrophoresis to separate the backbone vector from the EBNA-LP cDNA fragment. The backbone vector (9265bp) was then purified and ligated with the AvrII/BglII EBNA2 containing fragment to generate the EBNA2 shuttle vector. Figure 4.14B shows the BamHI/FspI and XbaI digest of the EBNA2 shuttle vector, which gave bands of the predicted sizes confirming the construction of the shuttle vector was correct.

To confirm that the EBNA2 shuttle vector was capable of expressing EBNA2, DG75 was transiently transfected with the EBNA2 shuttle vector, pSG5 (negative control) and pSG-EBNA2 (positive control) and EBNA2 expression was detected by immunofluorescence cell staining as previously described. Representative EBNA2 stains (red) and DAPI

counterstains (blue) of the same fields are shown in Figure 4.15, confirming that the shuttle vector could express EBNA2.

After confirmation of the integrity of the EBNA2 shuttle vector, homologous recombination was used to introduce the EBNA2 cDNA into the 2W BAC genome with the hygromycin resistance gene and CMV promoter regions acting as the homologous regions on either sides of the cDNA (Figure 4.16). Instead of using the rec A^+ BJ5183 *E.coli* strain to carry out the recombination, we used the 2W containing DH10B (rec A^-) bacteria transformed with a pDK46 plasmid, which contains the λ-red recombinase under the control of an arabinose-inducible promoter and a temperature-sensitive replication origin. These bacteria were grown at 30ºC in medium containing L-arabinose to induce the expression of the recombinase before being made electrocompetent. The EBNA2 shuttle vector was linearised by digestion with FspI which cut at a single site in the vector and electroporated into the 2W/pDK46 containing DH10B. Recombinants were selected on chloramphenicol/tetracycline agar plates, grown at 42ºC to induce loss of the pDK46 plasmid and screened by BamHI restriction enzyme digestion. A correct clone was identified and the BAC DNA was then re-checked by restriction enzyme digestion with AvrII, EcoRI and NheI in addition to BamHI. Figure 4.17 shows these restriction profiles of BamHI, AvrII, EcoRI and NheI digests in comparison to the parental 2W BAC, which confirmed that the 2W-EBNA2 BAC was correct. The complete 2W-EBNA2 BAC was then transfected into 293 cells and stably transfected clones were selected with hygromycin B, expanded and screened for virus production as previously described.

4.3.2 Infection of resting B cells with 2W-EBNA2 repaired virus

To analyse the effect of additional expression of EBNA2 on B-cell transformation, a preliminary experiment was carried out for binding (Q-PCR), antigen expression (IF staining) and transformation efficiency (transformation assays) comparing the 2W-EBNA2 virus with the 2W, 2W-LP and 5W virus. First, Figure 4.18 shows the result of Q-PCR assays measuring the amount of each virus initially bound to the cells. In this particular assay, approximately 2 fold less 2W-EBNA2 virus bound to B cells compared to 2W, 2W-LP and 5W viruses.

In the same experiment, infected cells were then monitored for EBNA-LP and EBNA2 protein expression by immunofluorescence cell staining (Figure 4.19A). EBNA-LP and EBNA2 positive cells (red) and the total number of DAPI positive cells (blue) were counted on 4 fields of at least 100 cells to determine the percentage of EBV antigen-expressing cells (Figure 4.19B). The percentages of EBNA-LP and EBNA2 positive cells in 2W-EBNA2 repaired virus infected cultures (11.9% and 11.2%, respectively) were even lower than those in parental 2W infected cultures (EBNA-LP: 22.2% ; EBNA2: 23.3%). However, higher percentages of antigen-positive cells were detected in cultures infected with the 2W-LP repaired virus (EBNA-LP: 38.2% ; EBNA2: 40.4%) compared to 2W virus, although still much lower than for 5W (EBNA-LP: 82%; EBNA2: 68.5), confirming the previous result (Figure 4.12A). In this particular infection experiment, more antigen expressing cells were detected overall compared to the previous experiments (Figure 4.12A).

Transformation assays as the ultimate read out for transformation efficiency were also carried out in the same experiment. Figure 4.20 shows the results of the transformation assays. Similar transformation efficiencies were observed in 2W and 2W-EBNA2, while 2W-LP virus showed a higher efficiency, the level was still lower than 5W viruses. Thus, the m.o.i. for 50% transformation was 10.8 and 14.2 for 2W and 2W-EBNA2, respectively, 3.4 for 2W-LP and only 0.5 for 5W virus.

It should be noted that B cells used in this preliminary experiment was isolated from cones, and better infection of 2W, 2W-LP and 5W viruses were observed in this experiment compared to the previous similar experiment (Figure 4.12 and Figure 4.13.) using B cells isolated from buffy coats. Importantly, although the absolute numbers were higher in this experiment, the trend we saw in this experiment was exactly the same as in the previous experiment. In this preliminary experiment for the 2W-EBNA2 virus, no clear effect was observed. However, this could be due to the poor virus binding (Figure 4.18), and presumably low internalisation with 2W-EBNA2.

In a subsequent experiment, Western blotting was used to detect the expression of EBNA-LP and EBNA2 in cells infected with 2W-EBNA2 and 2W viruses. As shown in Figure 4.21, higher levels of EBNA2 and EBNA-LP expression were observed in cells infected with the 2W-EBNA2 virus compared to cells infected with the 2W virus.

4.4 Discussion

The immediate background to these studies was the earlier finding (Chapter 3) that BamHI W repeat number had a major influence on EBV B cell transforming function. In particular, 0W and 1W viruses had no detectable transforming ability, while 2W virus was around 100 fold less active than the optimum (shown by virus with ≥ 5 W copies) with 3W/4W viruses showing intermediate activities. This may reflect the fact that when Wp copies are reduced in number below 5, Wp-initiated transcription is limiting in freshly-infected B cells. However another consequence of reducing BamHI W repeat number is to reduce the size of one of the key latent proteins, EBNA-LP, whose repeat sequence is encoded by the W1 and W2 exons within each BamHI W repeat. Thus the 2W virus is predicted to make an EBNA-LP with two copies of the W1W2 repeat domain.

In order to distinguish between an effect of reducing Wp number itself and the knock-on effect from reducing EBNA-LP size, the following approaches were attempted:-

1. to use the E2KO and LMP1KO as helper virus for EBV with limiting numbers of BamHI W repeats
2. to insert full length EBNA-LP or EBNA2 cDNA into the poorly transforming 2W EBV genome.

Helper virus experiments

Initially, characterisation of the helper viruses was carried out by infecting B cells with either the E2KO or the LMP1KO virus alone and monitoring RNA transcription by Q-RT-PCR (Wp- and Cp-initiated transcripts and BHRF1 and EBNA2 transcripts) and protein

expressions by Western blotting (BHRF1, EBNA-LP, 1, 2, 3A, 3C and LMP1) (Figure 4.1 and Figure 4.2).

In Chapter 3, we had shown that with wild-type virus, Wp activity peaked within the first 24 hours post infection, followed by a rapid drop and then a more gradual decline, while Cp activity became apparent by 48 to 72 hours post infection. Surprisingly, completely different kinetics were observed for E2KO infection. Wp activity increased to very high level and remained high for at least 72 hours post infection. Likewise a progressively increasing level of BHRF1 was detected during the 72 hours time course mirroring this sustained Wp activity; and therefore reinforcing the connection between Wp activity and BHRF1 first suggested by Kelly et al. (Kelly et al., 2009). At the same time, Cp initiated transcription from E2KO virus was detectable from 24 hours onwards but by 72 hours only matching the very low level (< 2% of 5W virus) shown by the 2W virus itself. This reinforced the previous studies showing that Cp activation requires transactivation by EBNA2 (alone or with EBNA-LP) (Harada and Kieff, 1997, Jin and Speck, 1992, Schlager et al., 1996, Yoo and Speck, 2000), and that EBNA1 is not sufficient to transactivate Cp in the absence of EBNA2 (Nilsson et al., 2001, Schlager et al., 1996). The fact that Wp levels remained high up to 72 hours post infection in the absence of significant Cp activation is also consistent with the idea of Cp transcript-mediated interference with Wp activity (Puglielli et al., 1997, Puglielli et al., 1996, Yoo et al., 1997).

Very interestingly, from the immunoblots, full-length EBNA-LP, EBNA1, EBNA3A and EBNA-3C were strongly expressed by the E2KO virus even within 24 hours post infection,

while BHRF1 (usually detectable at the protein level in early post infection) was also detectable by day 1 and became strongly expressed by day 3-7. Referring to the transcription data at the same time points, these proteins detected at one day post infection must be being expressed from Wp, rather than Cp; furthermore the same is probably true for the proteins at least up to day 3. Unexpectedly, LMP1 was also detected in E2KO virus-infected cells at 3 days post infection. Although we do not know which promoter was used to express LMP1 at this time, recent studies showed that NF-κB can transactivate the LMP1 promoter (ED-L1 promoter) in the absence of EBNA2 (Johansson et al., 2009, Demetriades and Mosialos, 2009). Nevertheless, even though E2KO virus produced all the latent proteins except EBNA2 as published, it still never drove the infected B cells into cell cycle and failed to induce B-cell transformation (Altmann and Hammerschmidt, 2005, Tierney et al., 2007). This emphasises the essential role of EBNA2 in B-cell transformation (Cohen et al., 1989, Hammerschmidt and Sugden, 1989, Kempkes et al., 1995c). These results showed that, for our purposes, E2KO can be used as a transformation-defective helper to try and rescue the impaired 2W virus by providing large amount of a sufficiently large EBNA-LP in early infection.

In B cells infected with the LMP1KO virus, just as in wild-type EBV infection, Wp activity peaked within the first 24 hours post infection and then gradually decreased, while Cp activity was induced later and became apparent by 48 to 72 hours post infection. EBNA2 transcripts showed similar kinetics to Wp activity. While, by Western blotting, a large amount of EBNA-LP and EBNA2 were detected at one day post infection; whereas EBNA1, EBNA3A and EBNA3C were detected at three days post infection; this is again

typical of the kinetics seen in wild-type EBV infection, the key difference being that no LMP1 was expressed (Dirmeier et al., 2003). Also for reasons that are not clear, BHRF1 transcription was significantly lower for the LMP1KO virus than for the E2KO virus and BHRF1 protein was not detectable. Most importantly, in the context of the planned helper experiments, the LMP1KO virus made easily detectable levels of EBNA-LP species with 3-6 W1W2 repeats, which although smaller than the E2KO virus encoded EBNA-LP, should still be sufficient for wild-type EBNA-LP activity. Note also that microscopic examination of cultures of B cells infected with the LMP1KO virus showed blast transformation and cell growth occurring for approximately 2 weeks post infection; after that in the absence of fibroblast feeder cells, no LCLs grew out and cultures eventually died (Dirmeier et al., 2003). Thus LMP1 is not essential for the initiation of B cell growth transformation but is essential for continued growth and LCL establishment. On this basis, we predicted that the LMP1KO virus would be a better helper than the E2KO virus, providing both EBNA2 and a sufficiently large EBNA-LP in trans while itself being non-transforming.

The effect of these helper viruses on 1W and 2W virus infections was first analysed by Q-RT-PCR. It is difficult for us to draw conclusions from these assays (Figure 4.2), because the helper viruses themselves dominate the Wp transcription and (in the case of LMP1KO virus) Cp transcription and also EBNA2 transcription. But importantly, the E2KO helper virus did not improve EBNA2 transcript levels for the 2W virus above their very low level, and its only effect on the 1W virus was to make EBNA2 transcription very weakly detectable at 72 hours. In the corresponding experiments, looking at help for transformation, the E2KO virus marginally improved transformation efficiency of 2W virus as previously

described (Tierney et al., 2007). This 2-fold increase in transformation efficiency may not be large enough to be reflected quantitatively in the transcription assays. By contrast, the LMP1KO helper virus significantly improved transformation efficiency of 2W, restoring it to a level similar to that of 5W virus in earlier work (Figure 4.3 and Figure 3.7) and was even able to provide sufficient help to achieve transformation in some wells using high doses of 1W virus. These findings are consistent with the hypothesis that sufficient levels both of EBNA-LP and of EBNA2 proteins are required to switch on Cp and drive the transformation process.

Repaired virus experiments

As made clear in Figure 4.1, a potential complication of the helper virus approach is that the E2KO and LMP1KO viruses also express other EBV latent antigens besides full-length EBNA-LP. As an additional approach, therefore, we attempted to repair the 2W virus first by introducing a 7 repeat EBNA-LP cDNA driven by CMV-IE promoter into the 2W virus genome at a site distant from Wp. Overall, this led to an approximately 2-fold improvement in latent gene transcription, latent protein expression and B cell transformation compared to the 2W virus. However, this EBNA-LP repaired 2W virus was still less active than the 3W virus and much less active than the 5W virus standard. It should be noted that, even though the EBNA-LP shuttle vector used during BAC construction did express the expected 7-repeat EBNA-LP protein, and the 7 repeat sequence had been recombined intact into the 2W genome, B cells infected with the 2W-LP virus expressed three different sizes of EBNA-LP equivalent to 2, 3 and 4 repeat EBNA-LP. Despite this, the EBNA-LP repair had the same effect on 2W virus infection/transformation as the E2KO helper virus, which

provided full-length EBNA-LP. This suggested that one of the factors limiting 2W virus activity was the level of EBNA-LP expression early post infection, and not necessary the size of the EBNA-LP protein per se. The fact that neither approach had a large effect on 2W activity, whereas the LMP1KO virus did have such an effect, indicated that EBNA2 expression was by far the most critical factor.

Therefore, in another approach to repair the 2W virus, we introduced CMV-driven EBNA2 into the 2W virus genome. Unfortunately, time did not allow this work to be completed. From one preliminary experiment, 2W-EBNA2 showed no increase in EBNA-LP and EBNA2 expression by immunofluorescence staining, and a transformation assay carried out with the same virus stocks and the same B cells showed no improvement compared to 2W virus. However, these results were difficult to interpret, since in this particular experiment, there was less 2W-EBNA2 virus binding to B cells than 2W virus. In a subsequent experiment using Western blotting, 2W-EBNA2 did show slightly higher level of both EBNA2 and EBNA-LP proteins than 2W virus and so more work is required before any conclusions can be made.

In this section of work, we tried to explain the impaired transformation ability of 2W virus. Was it the result of producing a truncated EBNA-LP which failed to function as a transactivator? Or were two copies of Wp insufficient to achieve optimal Wp-initiated transcription in freshly-infected resting B cells and therefore insufficient to produce enough EBNA-LP and EBNA2 to activate the Cp and LMP promoters and drive the cells into cell cycle? By providing additional EBNA-LP either from within the genome or from

coinfection with the E2KO helper virus, the impaired transformation ability in the 2W virus could not be restored fully to the wild-type virus level. In contrast, with the help of LMP1KO virus, which provided not only the high level of wild-type EBNA-LP, but also additional EBNA2, the impaired transformation ability of 2W virus was restored to wild-type levels. These results suggest that while EBNA-LP is important in the initiation of B-cell growth transformation, its full effect is only seen in the presence of EBNA2. We conclude that the viruses with a low number of BamHI W repeats are relatively impaired in transforming ability, not because of the size of their encoded EBNA-LP per se but because of low Wp-initiated transcription leading to suboptimal levels of EBNA2 and EBNA-LP co-expression. This is consistent with the model (described earlier) in which every Wp copy is potentially active in the early stages of B cell infection.

Chapter 5. The role of BSAP in Wp-regulation

Using electrophoretic mobility shift assays (EMSAs) and reporter constructs, two regulatory regions within Wp, UAS1 and 2, and the transcription factors that influence their activity have been identified (Bell et al., 1998). UAS2 contains two binding sites for the ubiquitous transcription factor YY1 and shows lineage-independent activity in reporter assays. By contrast UAS1 contains two binding sites for the B lineage-restricted BSAP protein, plus sites for the ubiquitous RFX and CREB/ATF transcription factors, and has B cell-specific activity (Bell et al., 1998, Kirby et al., 2000, Tierney et al., 2000a). To determine the importance of these regions in the physiological setting of the complete virus genome, BAC cloning was used to construct recombinant EBVs carrying two tandemly-arranged copies of Wp deleted either for UAS1 and/or UAS2 (Tierney et al., 2007). This showed that UAS2 was completely dispensable for Wp activation and subsequent B cell transformation, whereas UAS1 was absolutely essential for both activities. Subsequently a recombinant virus (2WBSAPm) was produced carrying two Wp copies in which both BSAP sites were inactivated. Like the UAS1 knockout, this virus showed greatly reduced Wp activity in resting B cells and did not transform B cells. Since the 2W virus itself has reduced transforming ability relative to the parental 2089 virus, in the third part of this thesis, we tried to test whether the apparent dependence of EBV transforming function upon BSAP sites in Wp in a 2W virus was still apparent when one increased the number of W copies.

5.1 Construction of the 5WBSAPm BAC

Having previously shown that 5 copies of the BamHI W are required for optimal transformation (Chapter 3), a recombinant virus carrying five copies of the BamHI W repeat in which both BSAP sites were inactivated was made. To generate this 5WBSAPm virus, an engineered 5WBSAPm shuttle vector was constructed in two parts. In the first part (Figure 5.1), a new shuttle vector was made by introducing a multiple cloning site (Reeves et al.) into pBS 5'3' (Tierney et al., 2007) between the sequences homologous to the BamHI C and BamHI Y fragments (i.e. sequences that are naturally 5' and 3' of the BamHIW repeats in EBV). Three pairs of oligonucleotides containing different multiple restriction sites (which do not cut within the BamHI W fragment) were first generated. Each pair of oligonucleotides was incubated at 95ºC for 5 minutes, cooled down gradually to allow annealing to each other. The first double stranded oligonucleotide was then ligated into BamHI-digested pBS5'3' vector to generate pBS 5'-MCS1-3'. The second double stranded oligonucleotide was then ligated into BsrGI/NotI digested pBS 5'-MCS1-3' vector to generated pBS 5'-MCS1+2- 3'. Finally, the third double stranded oligonucleotide was ligated into SbfI/NheI digested pBS 5'-MCS1+2- 3' to generate the final MCV vector (Figure 5.1).

In the second part of the cloning strategy (Figure 5.2), five BamHI W fragments with both BSAP sites inactivated (BSAPmW) were amplified from pBS 5'-BSAPmW-3' (Tierney et al., 2007) by PCR with primers containing restriction sites present in the MCS. These W

fragments were then digested with the appropriate restriction enzymes and cloned sequentially into MCS vector to generate the final 5WBSAPm shuttle vector.

To generate the 5WBSAPm BAC, homologous recombination was used to introduce the five copies of mutated BamHI W (5xBSAPmW) into the 0W BAC genome with the BamHI C fragment and BamHI Y fragment acting as the homologous regions on either side of the 5xBSAPmW (Figure 5.3). The 5WBSAPm shuttle vector was linearised by digestion with AhdI which cuts at a single site in the vector outside of the 5WBSAPm and homologous regions. The linearised DNA was then transformed into rec A^+ BJ5183 *E coli*. containing the 0W BAC plasmid. Recombinants were selected on chloramphenicol/tetracycline agar plates and were screened by EcoRI/HindIII restriction enzyme digestion. A correct clone was identified and the BAC DNA was then transformed into the rec A^- DH10B *E coli*. strain for propagation and maintenance. The BAC DNA was then re-checked by restriction enzyme digestion with ScaI, which cuts within one of the mutated BSAP binding sites. Figure 5.4 shows these restriction profiles of BamHI, EcoRI/HindIII and ScaI digests, with the red arrows indicating the bands containing the BamHI W repeats and blue arrows indicating the bands predicted difference between 5WBSAPm and 5W BAC. After confirming the integrity of the construct, 5WBSAPm BAC was then transfected into 293 cells to generate the virus producing cell line.

5.2 Infection of resting B cells with 5WBSAPm virus

To characterise this virus, a series of binding assays, transcription assays, IF cell staining assays and transformation assays were then carried out comparing the 5WBSAPm virus to the 5W virus and the previously published 2WBSAPm virus (Tierney et al., 2007).

Figure 5.5 shows the result of Q-PCR assays from one representative experiment measuring the amount of each virus initially bound to the cells and the result demonstrated that 5WBSAPm virus bound to B cells with equal efficiency as 5W virus. This experiment was carried out 2 times independently and similar results were observed in each experiment.

Q-RT-PCR analysis of Wp-initiated, BHRF1, EBNA2 and Cp-initiated transcripts from one preliminary experiment is shown in Figure 5.6. In line with the previous studies (Tierney at al. 2007), no transcription was detected in the 2WBSAPm virus. Interestingly both Wp and Cp activity and BHRF1 and EBNA2 transcripts were detected in 5WBSAPm infected cells. However, in comparison to 5W virus, the level of transcription was much lower with 5WBSAPm.

EBNA-LP and EBNA2 protein expression were monitored by immunofluorescence cell staining. B cells infected with the 2W, 2WBSAPm, 5WBSAPm and 5W viruses at an m.o.i of 50 were harvested at day 3 post infection and stained for EBNA-LP and EBNA2. EBNA2/EBNA-LP positive cells (red) were counted against the total number of DAPI stained cells (blue) on 4 fields to determine the percentage of EBV antigen-expressing cells. As shown in Figure 5.7, 22.2% cells were EBNA-LP positive and 23.3% were EBNA2 positive in the culture of 2W infected cells, while as expected, no EBNA-LP or EBNA2 were detected in 2WBSAPm infected cells. A higher percentage of cells was found to express EBNA-LP and EBNA2 in cultures infected with the 5W virus (EBNA-LP: 82% ; EBNA2: 68.5%) compared to 5WBSAPm virus (EBNA-LP: 36.4% ; EBNA2: 43.4%).

To directly quantitate the transformation efficiencies of the 5WBSAPm virus compared to the 2W, 2WBSAPm and 5Wvirus, transformation assays were also carried out in the same experiment. Figure 5.8A shows the results of the transformation assays plotted as bar charts. No transformation was observed in the 2WBSAPm virus, which confirmed the observation in the previous studies (Tierney et al., 2007). In line with previous data (Chapter 3), 2W virus showed lower level of transformation efficiency compared to 5W virus. Most interestingly, similar transformation efficiencies were observed in 5WBSAPm virus in comparison to the 5W virus. Figure 5.8B shows the data from the same experiment plotted as log (m.o.i.) against number of wells transformed. For 2W, the m.o.i. for 50% transformation was 4.4 and was higher than the m.o.i for 5W (m.o.i: 0.09) virus and for 5WBSAPm virus (m.o.i: 0.05).

5.3 Discussion

In this phase of work, we were trying to address the importance of B-cell specific transcription factors binding within Wp in the context of viral infection. In particular, sequences upstream of Wp have been shown to be important for Wp-initiated transcription (Puglielli et al., 1997, Ricksten et al., 1988, Woisetschlaeger et al., 1989) and, in some cases, Cp-initiated transcription (Ricksten et al., 1988). Bell et al. have identified the presence of three regulatory regions: UAS1 which exhibits B-cell-specific activity, and UAS2 and UAS3 which exhibit cell lineage-independent activity (Bell et al., 1998). Subsequently, it was shown that UAS2 contains two YY1 sites and UAS1 contains CREB,

RFX and two BSAP binding sites (Kirby et al., 2000, Tierney et al., 2000a) and of these factors, only BSAP is B-cell specific.

In the literature, two studies have shown the importance of the regulatory regions of the Wp promoter for B-cell growth transformation (Tierney et al., 2007, Yoo et al., 2002). In the previous studies, Yoo et al. attempted to make recombinant viruses with a 200bp deletion (from -369 to -169 bp) upstream of the first copy of Wp (Wp1), that removed both YY1 sites in UAS2 and BSAP binding site D in UAS1. To achieve this, a combination of two constructs, one to repair the EBNA2 deletion, and the other containing two copies of BamHI W fragment with the 200bp deletion in the first copy, were transfected into the non-transforming EBV strain P3HR1, along with a BZLF1 gene expression vector to induce virus replication. The resulting heterogeneous virus stock was then used to infect primary B cells. All LCLs which arose from this infection were screened and analysed to determine where the mutant Wp recombined within the BamHI repeat. Among all the 253 LCLs, most LCLs were immortalised by recombinant viruses harbouring the 200bp deletion downstream of Wp1 (ie. in Wp2 or Wp3) and only a single LCL was immortalised by a recombinant EBV harbouring the deletion upstream of Wp1. The very poor transforming ability of virus with this deletion in the first Wp copy, considered to be the most active in an LCL, suggests that this region of Wp is essential for B-cell growth transformation (Yoo et al., 2002). The limitations of this study were firstly that it relied on growth transformation as the read-out and secondly that wild-type BamHI W repeats were still present in the recombinant viruses generated.

In the later study, the B95.8 based system was used to generate a series of recombinant viruses containing two copies of BamHI W fragments in which either the UAS1, UAS2 or UAS1 and UAS2 regions of Wp had been deleted or the BSAP-binding sites in UAS1 had been mutated (Tierney et al., 2007). This approach had the advantage that pure virus stocks containing the desired mutation in both BamHI W repeats could be made in the absence of any wild-type BamHI Ws. In addition, this virus could be used to directly infect resting B cells and look at early EBV transcription and gene expression in addition to the late B-cell transforming ability. Recombinants with UAS1 deleted failed to initiate viral transcription and transform B cells, while deletion of UAS2 had no detectable effect on the virus's transforming function. Most importantly, recombinants with mutation of the two BSAP binding sites in UAS1 also lost all detectable transcriptional and transforming activity. During lymphocyte ontogeny BSAP is first expressed at the multipotent pro-B-cell stage and actually drives the final commitment to the B-cell lineage (Matthias and Rolink, 2005). Our hypothesis is that the UAS1-binding protein, BSAP/Pax5 is a key determinant of the B cell lineage specificity of EBV's growth transforming programme.

However, it should be noted that the previous BSAP mutant virus only contained two copies of BamHI W fragments, and the work presented in this thesis (Chapter 3) and previous studies (Tierney et al., 2007) have shown that 2W virus itself is impaired for its transformation ability. Therefore, in order to further investigate the importance of the Wp BSAP binding sites in B-cell-transformation, we constructed a mutant virus carrying five BamHI W repeat fragments with mutant BSAP binding sites in every repeat (5WBSAPm). This was based on our previous results showing that five copies of BamHI W were

sufficient for optimal transformation efficiency. Therefore the mutation could be tested in a virus genome which was approximately 100 times more efficient than 2W virus itself. To achieve this, linkers containing multiple cloning sites were introduced into a shuttle vector to allow us to sequentially clone in BamHI W fragments containing the mutated BSAP binding sites. Importantly, this system allows one not only to introduce any desired mutation into every BamHI W copy, but also to generate viruses carrying different numbers of mutated BamHI W repeats.

In preliminary experiments, 5WBSAPm virus showed a significant reduction of Wp- and Cp-initiated transcription and BHRF1 and EBNA2 transcription in comparison to 5W virus. We also observed less expression of EBNA-LP and EBNA2 with 5WBSAPm virus by IF staining relative to the expression with 5W virus. Unexpectedly, however, in a separate transformation assay, 5WBSAPm virus showed similar transformation efficiency to 5W virus. These results suggest that mutation of the BSAP binding sites may have an effect on viral transcription. It is possible that the effect of mutation in the BSAP binding sites is more subtle in the context of a 5W virus than in the poorly transforming 2W virus. Unfortunately, the limited data obtained are insufficient for us to make any conclusion at present. Work is still currently progressing to test out the effects of BSAP mutation in the whole spectrum of 2W, 3W, 4W and 5W viruses using the constructs made during this thesis.

Chapter 6. Final discussion and future work

As a B lymphotropic virus, EBV's biology is closely entwined with the biology of B lymphocytes. To establish and maintain persistence *in vivo*, EBV uses different transcription programmes at different stages of the virus lifecycle, which may be dependent on the differentiation stages of the host cell. We believe that the virus initially activates and drives the growth of newly infected B cells, using the growth transformation programme. Wp/Cp initiated transcripts, characteristic of this latency III growth transformation programme, have been detected in the peripheral blood mononuclear cells (PBMC) of infectious mononucleosis (IM) patients undergoing primary infection (Tierney et al., 1994) and EBNA2 expression (Cp or Wp driven) has also been detected in tonsillar B cells from IM (Niedobitek et al., 1997a). *In vivo* infection of host cells most likely involves a very low dose of virus. Therefore it is essential that the virus drives the infected cell into cycle to amplify the pool of infected cells in the host, before the host immune system is activated. Our hypothesis is that the multiple copies of Wp serve to initiate high levels of viral gene transcription in the context of a quiescent cell, thus producing high levels of EBNA-LP and EBNA2 which then activate both additional viral and cellular promoters, which in turn drives the cell into cell cycle.

The work described in this thesis uses *in vitro* model of primary B cell infection. Using recombinant EBV viruses carrying different numbers of BamHI W repeats, we have shown

that virus with more BamHI W repeats (ie. more copies of Wp) had a higher level of Wp activity, produced more EBNA-LP and EBNA2 protein and transformed B cells more efficiently. The virus requires at least 2 copies of the BamHI W repeat to be able to initiate the growth transforming programme in B cells *in vitro*, and at least 5 copies of BamHI W for optimal transformation. These *in vitro* studies support the notion that in the quiescent environment of a resting B cell, a high level of Wp-initiated transcription is required to initiate the growth transformation programme and to achieve this the virus has acquired multiple copies of BamHI W repeats, and therefore multiple copies of Wp promoter, during virus evolution.

This hypothesis has been reinforced by the observation that large internal repeat regions, homologous to BamHIW are present in other lymphocryptoviruses. For example, the Old World rhesus LCV contains an IR1 comprised of 5.7 copies of a 3072bp repeat sequence and the New Word LCV, marmoset LCV prototype contains an IR1 comprised of 7.5 copies of a 2912bp repeat sequence (Rivailler et al., 2002a, Cho et al., 2001). Most importantly, the Wp sequence in the rhesus LCV is more than 80% identical to the EBV Wp (Peng et al., 2000a), and the Wp sequence in the marmoset LCL is more than 60% identical to the EBV Wp (Rivailler et al., 2002a). The existence of this conserved promoter containing, large internal repeat both in the genome of the New World and Old World LCVs and in EBV in man, suggests that the amplification of the BamHI W fragment occurred early in primate evolution and is an essential component of the virus.

Wp activation and the B cell growth transforming programme are clearly important during the early stages of B cell infection. However the transforming programme is not compatible with the establishment of a persistent latent infection *in vivo* as most of the genes expressed in latency III are targets of the host immune system. The virus must therefore down-regulate expression of most of its latent genes to become immunologically silent, and as such only the EBER and BART RNAs are detectable in the PBMCs of healthy virus carriers (latency 0). Studies have suggested that methylation and chromatin re-modelling of the EBV genome are involved in transcriptional silencing (Hutchings et al., 2006). However at present there is no *in vitro* model for this latency 0 form of infection.

It would be instructive therefore, to use an animal model to study the role of the BamHI W repeats and Wp during primary infection and the establishment of persistent infection. Using an animal model, we could analyse virus transcription immediately after infection; samples obtained from IM patients, who are already displaying symptoms of infection, represent later time points of infection where the immune response has already been activated. Also by using our nW viruses, we could compare the virus ability to initiate the growth transformation programme and also the ability on establishing persistence in the host. There are two options of animal model systems we could possibly use. The first is the mouse model that partially reconstitutes the human immune system component after engraftment of hematopoietic progenitor cells (HPC) (Strowig et al., 2009, Ishikawa et al., 2005). Purified virus particles could then be injected in to the humanised mice intraperitoneally, and samples would then be collected and analysed post infection. Alternatively, we could choose the rhesus macaques model using rhLCV BAC (Chen et al.,

2005a). The identical repertoire of lytic and latent infection viral genes in the rhLCV and EBV genomes provides important genetic validation that rhLCV is an accurate animal model for EBV infection (Rivailler et al., 2002b, Wang, 2001). To use this system, we would need to generate the "nW" virus containing different numbers of the 3072bp motif, homologous to the BamHI W repeat using the rhLCV BAC system.

In our *in vitro* model, we observed that virus required at least two copies of the BamHI W repeat to initiate the growth transformation programme. It should be noted that, the *in vitro* infection was carried out with high dose of virus stocks, therefore in the more complicated host environment and with much lower virus dose, it is even more essential for virus to establish the growth transformation programme to be able to establish persistence. Thus, virus may require more than 2 copies of BamHI W (ie. two copies of Wp) to initiate sufficient level of transcription and to transform the cells. In the animal model experiments, after infection, samples (saliva, tonsil, blood etc.) would be collected from different time points post infection to carefully monitor the activity of the viral growth transformation programme, including promoter usage, viral antigen expression at the early and later time points. The virus load in the host could also be used as a read-out for the efficiency of the virus infection. This animal model could provide us a clearer picture of the role of the BamHI W repeat and multiple Wp copies in the growth transformation programme *in vivo* in primary infection and persistence.

From our previous experiments of using the E2KO helper virus or the 2W-LP to provide additional EBNA-LP, we could only marginally improve B cell transformation with the

impaired 2W virus. Furthermore, by coinfection with the LMP1KO helper virus, which provided both EBNA2 and EBNA-LP, we could improve the transformation ability to optimal level. Therefore, we concluded that the key function of high level Wp-initiated transcription was to produce sufficient level of both EBNA-LP and EBNA2 expression which then serves to activate Cp and other viral promoters, along with cellular promoters for subsequent stages of the growth transformation programme.

To further test our hypothesis, it would therefore be instructive to construct a single 2W virus repaired for both EBNA2 and EBNA-LP expression. This could potentially be done in several different ways. We could repair the 2W-LP by placing the EBNA2 encoding cDNA downstream of the IRES sequence following the LP cDNA, or vice versa, we could replace the EBNA-LP cDNA downstream of the IRES in the 2W-EBNA2 virus. However it is likely that in using either of these approaches that the gene downstream of the IRES would be expressed at a lower level than the upstream gene. An alternative method would be to construct an entirely new construct in which we were able to express these two proteins independently from a single promoter using the "self-cleaving" 2A (T2A) peptide sequence placed between EBNA-LP and EBNA2. If our hypothesis is correct, we would then expect this repaired virus (2W-EBNA-LP/EBNA2) to achieve optimal transformation in resting B cells.

The studies presented in this thesis provide some insight into the mechanism EBV uses to achieve the growth transformation programme in the quiescent environment of a resting B

cell. EBV initiates the growth transformation programme through the activation of a multimerised promoter, Wp, which is solely dependent on cellular transcription factors for its activation (Bell et al., 1998, Kirby et al., 2000, Tierney et al., 2000a). Transcription from the multiple Wp copies, which are all active immediately post-infection, produces large amounts of EBNA-LP and EBNA2, which activate viral and cellular promoters and drives the infected cell to proliferate, thus increasing the population of infected cells. Activation of a host immune response against the latent gene products expressed in this growth transforming programme will ultimately select for EBV infected cells which have down regulated viral expression (latency 0) and a stable virus/host balance will be established.

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